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PATENT

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10/19/06 Michelle Hobson

Date Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: ZHANG et al. Serial No.: 09/464,795 Filing Date: December 16, 1999 Title: NON-INVASIVE EVALUATION OF PHYSIOLOGICAL RESPONSE IN A MAMMMAL	Examiner: Anne Marie Falk Group Art Unit: 1632 Confirmation No.: 8087 Customer No.: 20855

TRANSMITTAL

Mail Stop Appeal Brief
Commissioner for Patents
P. O. Box 1450
Arlington, VA 22313

Dear Sir:

Transmitted herewith for filing are the following documents in the above patent application.

X Revised Appeal Brief (17 pages) with attached Claims Appendix (4 pages), Evidence Appendix (1 page) and Related Proceedings Appendix (1 page)

X Reply Brief (23 pages) with attached Claims Appendix (4 pages), Evidence Appendix (1 page) with 4 attached documents and Related Proceedings Appendix (1 page)

X A return receipt postcard

The fee is calculated as follows:

	NO. OF CLAIMS	CLAIMS PREVIOUSLY PAID FOR	EXTRA CLAIMS	RATE	FEE
Total Claims	10	- 64	0	x $50.00	$0
Independent Claims	1	- 7	0	x $200.00	$0
Multiple dependent claims not previously presented add $360.00					$0
Total Amendment Fee					$0
Petition for Extension of Time Fee					$0
Fee for filing an Appeal Brief was previously submitted on June 1, 2006					$0
TOTAL FEE DUE					$0

The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 18-1648.

Respectfully submitted,

Date: October 19, 2006

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:	Examiner: Anne Marie Falk
ZHANG et al.	Group Art Unit: 1632
Serial No.: 09/464,795	Confirmation No.: 8087
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Title: NON-INVASIVE EVALUATION OF PHYSIOLOGICAL RESPONSE IN A MAMMAL	

REVISED APPEAL BRIEF

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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REVISED APPEAL BRIEF

Mail Stop Appeal Brief
Commissioner for Patents
Alexandria, VA 22313

Sir:

INTRODUCTION

Appellant submits one copy of this brief on appeal in accordance with Section 41.37 (69 Fed. Reg. 49962, Aug 2004). An Appeal Brief was submitted on June 1, 2006. An Examiner's Answer was mailed on August 20, 2006. A Notice of Non-Compliant Appeal Brief was mailed on September 19, 2006, making a revised Appeal Brief due on or before October 19, 2006. Accordingly, this revised Appeal Brief is timely filed. In addition, this revised Appeal Brief is being filed concurrently with a Reply Brief.

I. REAL PARTY IN INTEREST

Xenogen Corporation, the assignee of record of the above-referenced patent application, is the real party in interest in this matter.

II. RELATED APPEALS AND INTERFERENCES

Appellants are not aware of any related appeals, interferences or judicial proceedings.

III. STATUS OF THE CLAIMS

Claims 38, 40, 41, 45, 46, 49 and 65-68 are currently pending in the above-referenced case (hereinafter "the application") as shown in the Claims Appendix. Claims 1-37, 39, 42, 43, 44, 50-64 and 69-80 were canceled. Therefore, pending claims 38, 40, 41, 45, 46, 49 and 65-68 are appealed and stand rejected under 35 U.S.C. § 101 and 35 U.S.C. § 112, 1st paragraph (written description and enablement).

IV. STATUS OF THE AMENDMENTS

In response to the Examiner's Final Office Action mailed October 10, 2005, Appellants filed a Response with arguments and amendments to claim 38. An Advisory Action was mailed on April 28, 2006 indicating that the amendment would be entered and overcame the rejections under 35 U.S.C. § 112, 2nd paragraph. Thus, all claims on appeal (claims 38, 40, 41, 45, 46, 49, and 65-68) are rejected under 35 U.S.C. § 101 and 35 U.S.C. § 112, 1st paragraph (written description and enablement) for the reasons set forth in the Final Office Action and Advisory Action.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The claimed subject matter is drawn to transgenic mice comprising multiple light-emitting reporter constructs (page 4, lines 24-26) and to non-invasive methods of using these

transgenic mice, for example to determine the effect of analyte on the promoters of the reporter constructs (page 5, lines 7-19); to detect a level of expression in response to an analyte (page 5, line 20-22); and/or to screen a selected analyte (page 5, lines 17-19).

In particular, **independent claim 38** is drawn to a transgenic mouse comprising a panel of expression cassettes (page 4, lines 24-26). The transgenic mouse is produced by a method comprising the steps of introducing, into a mouse at an embryonic stage (page 60, lines 2-7; page 65), first and second expression cassettes (page 4, lines 1-5). The first expression cassette comprises a first promoter operably linked to sequences encoding a first light generating polypeptide (page 4, lines 2-3) and the first promoter is derived from derived from a first stress-inducible gene (page 4, line 17). The second expression cassette comprises a second promoter operably linked to sequences encoding a second light generating polypeptide (page 4, lines 4-5). The second promoter is derived from derived from a different stress-inducible gene than the first promoter is derived from (page 4, lines 6-8 and line 17).

Dependent claim 40 is drawn to a method of determining the effect of an analyte on gene expression mediated by promoters derived from stress-inducible genes, by administering the analyte to a living transgenic mouse of independent claim 38 under conditions that permit light generation mediated by said light generating polypeptide in the transgenic mouse, and determining the effect of the analyte on expression of the light generating polypeptide in a living transgenic mouse wherein said expression is mediated by at least one of the promoters (page 5, lines 7-19).

Dependent claim 41 specifies that the conditions that permit light generation mediated by the light generating polypeptide includes administering, to the transgenic mouse, at least one substrate for the light generating polypeptide.

Dependent claim 45 is drawn to a noninvasive method for detecting a level of expression in response to an analyte, wherein said expression is (i) mediated by promoters derived from stress-inducible genes, and (ii) in a living transgenic mouse (page 5, lines 20-22). The method comprises (a) administering the analyte to a living transgenic mouse of claim 38, wherein administering of said analyte is carried out under conditions that permit light generation

mediated by said light generating polypeptide (page 5, line 24-25); (b) placing the transgenic mouse within a detection field of a photo detector device (page 5, lines 25-26); (c) maintaining the transgenic mouse in the detection field of the device (page 5, lines 26-27); and (d) during said maintaining, measuring photon emission from the transgenic mouse with the photo detector device to detect the level of expression of the light generating polypeptide in the living transgenic mouse wherein said expression is mediated by at least one of the promoters (page 5, lines 26-29). Dependent claim 46 specifies that steps (b) through (d) are repeated at selected intervals such that changes in the level of the light emission in the transgenic mouse are detected over time (page 5, lines 29 to page 6, line 3).

Dependent claim 49 is drawn to a method of providing a transgenic mouse suitable for screening a selected analyte, comprising generating a transgenic mouse of claim 38, and providing said transgenic mouse or progeny thereof for use in screening a selected analyte (page 5, lines 17-19).

Dependent claim 65 is drawn to the transgenic mouse of claim 38, and further specifies that the method comprises introducing a third expression cassette (page 4, line 11) comprising a promoter derived from a third stress-inducible gene (page 4, line 17) into a mouse at an embryonic stage, said third promoter operably linked to sequences encoding a third light generating polypeptide and said third promoter derived from a different stress-inducible gene than said first and second promoters (page 4, line 11-17).

Dependent claim 66 is drawn to the transgenic mouse of claim 65, wherein (i) said first, second, and third promoters are each derived from a different gene (page 4, line 6-8), and (ii) said first, second, and third light generating polypeptides produce the same color of light (page 4, lines 6-9).

Dependent claim 67 is drawn to the transgenic mouse of claim 65, wherein (i) said first, second, and third promoters are each derived from a different gene (page 4, line 6-8), and (ii) at least two of said first, second, and third light generating polypeptides produce different colors of light (page 4, lines 6-8).

Dependent claim 68 is drawn to the transgenic mouse of claim 65, and further indicates that the panel of expression cassettes comprises additional expression cassettes (page 4, lines 11-12), wherein each expression cassette comprises a promoter derived from a different stress-inducible gene, said promoter operably linked to sequences encoding a light generating polypeptide.

VI. GROUNDS OF REJECTION

1. Claims 38, 40, 41, 45, 46, 49 and 65-68 stand rejected under 35 U.S.C. § 101 as allegedly lacking patentable utility.

2. Claims 38, 40, 41, 45, 46, 49 and 65-68 stand rejected under 35 U.S.C. § 112, 1st paragraph as allegedly not adequately described by the specification as filed.

3. Claims 38, 40, 41, 45, 46, 49 and 65-68 stand rejected under 35 U.S.C. § 112, 1st paragraph as allegedly not enabled by the specification as filed.

VII. ARGUMENTS

1. Appealed Claims 38, 40, 41, 45, 49 and 65-68 Have Patentable Utility

Claims 38, 40, 41, 45, 46, 49 and 65-68 remain rejected under 35 U.S.C. § 101 as allegedly lacking patentable utility. (Advisory Action, page 2). In support of this rejection, the Advisory Action states:

> With regard to the utility rejection, Applicants assert, at pages 5-6 of the response, that the Examiner acknowledges there is well-established utility set forth in the specification. On the contrary, the Examiner alerted Applicant that the utility they were referring to was actually an **asserted** utility. ... An asserted utility should be not be confused with a well-established utility. ... An asserted utility must be specific to the claimed subject matter. The specification is clear that the intention is to create a construct and experimental system that recapitulates **native gene expression**, not gene expression in an artificial context.

> The specification does not provide specific guidance for creating constructs, within the scope of the claims that have this utility.[1]

Appellants reiterate the evidence of record establishes that the as-filed specification sets forth patentable utilities (well-established, credible and substantial) for the particularly claimed subject matter.

(a) Transgenic Mice Containing Reporter Constructs "Recapitulate Native Gene Expression"

The Examiner's utility rejection is based on the erroneous assertion that the asserted utility of "recapitulating native gene expression" is not supported in the as-filed specification.

In fact, "recapitulating native gene expression" is simply another way of phrasing the well-established use of reporter constructs to analyze gene expression. At the time of filing (and to this day), reporter constructs containing a promoter of a gene of interest operably linked to a reporter sequence were the preferred way of "recapitulating native gene expression," both *in vivo* and *in vitro*. The well-established use of reporter constructs to recapitulate native gene expression is described in the as-filed specification:

> Cardiovascular biology and diseases have been investigated in Tg mouse models using tissue-specific promoters [citations omitted] and regulation of insulin-responsive glucose transporter GLUT4 and Apo A-I genes have also been studied in models of diabetes, obesity [citation omitted] and coronary artery disease [citations omitted].
>
> Photoproteins as biological labels have been used for more than a decade for the study of gene expression in cell culture or using excised tissue [citations omitted]. Low-light imaging of internal bioluminescent signals has been used to study temporal and spatial gene regulation in relatively thin or nearly transparent organisms [citations omitted]. External detection of internal light penetrating the opaque tissues has been described [citation omitted].[2]

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[1] Advisory Action, page 2, emphasis in original

[2] Page 3, lines 2-33 of the application; *see, also;* page 22, lines 2-12 of the application

Thus, the asserted utility (non-invasive *in vivo* analysis of gene expression using animals comprising multiple reporter constructs) was well-established, substantial and credible for single constructs. As such, it is utterly irrelevant whether the "asserted" utility is phrased as "recapitulating native gene expression;" "analysis of gene expression;" or "use of reporter constructs to characterize the effect of an analyte on a particular promoter." The skilled artisan would view these terms as interchangeable and, moreover, would recognize the well-established, credible, substantial and specific utilities disclosed in the as-filed specification.

(b) The Actual "Asserted" Utility is Well-Established, Substantial and Specific

Moreover, as repeatedly noted, a utility rejection should not be imposed where there is a well-established utility and/or where there is one credible utility (*see,* M.P.E.P. § 2107, emphasis added):

> If at any time during the examination**, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility**. ...
>
> (1) If the applicant has asserted that the claimed invention is useful **for any particular practical purpose** (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, **do not impose a rejection based on lack of utility**.
>
> ... An applicant need only provide **one** credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement.

In the case on appeal, it was well-established at the time of filing that reporter constructs comprising a promoter operably linked to a reporter sequence "recapitulate native gene expression" (*i.e.,* via analysis of reporter gene expression which is indicative of the effect on the promoter operably linked to the reporter). Using a single reporter construct was (and remains) the preferred way to analyze gene expression which, in turn, facilitates, for example, drug

screening, both *in vitro* and *in vivo*.[3] This preferred method of promoter analysis (or, put in the Examiner's terms, the preferred method of recapitulating native gene expression) has a well-established utility and, accordingly, the rejection cannot stand.

In sum, the evidence of record demonstrates that reporter constructs recapitulate native gene expression by virtue of the promoter selected. Since this is a well-established, specific, credible and substantial utility for the claimed subject matter, Appellants submit that withdrawal of this rejection is in order.

2. **The As-Filed Specification Describes the Subject Matter of Appealed Claims 38, 40, 41, 45, 49 and 65-68**

Claims 38, 40, 41, 45, 46, 49 and 65-68 also remain rejected under 35 U.S.C. § 112 as allegedly not adequately described by the as-filed specification. (Advisory Action, page 3). In support of this rejection, the Advisory Action states:

> The [written description] rejection of record is grounded in the failure of the specification to describe those constructs that will provide for **native gene expression** and thereby be representative of native gene expression, such that the effect of an analyte on the construct will be representative of the effect of the analyte on the promoter (and its elements) in its native context. ... the specification has not described those constructs that will provide for **native gene expression**.[4]

For the reasons of record and noted above, Appellants submit that there is ample description in the specification, in view of what was well known in the art, regarding how reporter constructs as used in the claimed animals and methods allow for the screening of analytes via their effect on the promoter of the reporter constructs.

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[3] *See, e.g.,* Background and page 60, line 29 to page 61, line 6 of the application, describing the well known monitoring of expression of lux/luc reporter genes using non-invasive whole animal imaging and citing U.S. Patent No. 5,650,135.

[4] Advisory Action, page 3, emphasis in original

It is axiomatic that a patent specification "need not teach, and preferably omits, what is well known in the art." See, *Spectra-Physics, Inc. v. Coherent, Inc.* 3 USPQ2d 1737, 1743 (Fed. Cir. 1987); *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986). Thus, there is no requirement to describe that which is well-known at the time of filing. Indeed, this has recently been reiterated by the Federal Circuit in *Capon v. Eshhar* 76 USPQ2d 1078 (CA FC 2005):

> None of the cases to which the Board attributes the requirement of total DNA re-analysis, *i.e.*, *Regents v. Lilly, Fiers v. Revel, Amgen*, or *Enzo Biochem*, require a re-description of what was already known.
>
> It is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention. *See In re Angstadt*, 537 F.2d 498, 504 [190 USPQ 214] (CCPA 1976) ("The examples, both operative and inoperative, are the best guidance this art permits, as far as we can conclude from the record"). While the Board is correct that a generic invention requires adequate support, the sufficiency of the support must be determined in the particular case. ...

In the case on appeal, the state of the art and as-filed specification clearly establish that "recapitulating native gene expression" in transgenic animals by introducing a reporter construct comprising a selected promoter operably linked to a reporter sequence was well known at the time of filing:

> A wide range of Tg mice that employ reporter constructs have been developed and tested. For example, Tg mice containing the viral long terminal repeat (LTR) promoter fusions have been used to study the range of tissues and cells types that are capable of supporting HTLV-1 expression and the development of neurofibromatosis-like tumors associated with HTLV-1 retrovirus ... [citations omitted]. Cardiovascular biology and diseases have been investigated in Tg mouse models using tissue-specific promoters [citations omitted], and regulation of insulin-responsive glucose transporter GLUT4 and Apo A-I genes have also been studied in models of diabetes, obesity [citation omitted] and coronary artery disease [citation omitted].

> Photoproteins as biological labels have been used for more than a decade for the study of gene expression in cell culture or using excised tissue...[5] ...
>
> The monitoring of *lux/luc* reporter genes using non-invasive whole animal imaging has been described [citing U.S. Patent No. 5,650,135 and other citations].[6]

Thus, although not required because it is not new, the as-filed specification describes "recapitulating native gene expression" using a reporter construct was well known as well as how to make transgenic mice comprising these reporter constructs.

Furthermore, Appellants have described what *is* new, namely using panels of multiple reporter constructs in a single transgenic animal to allow analysis of multiple stress-inducible genes in a single live animal model:

> The present invention provides a powerful new tool for analyzing biochemical pathways and physiological functions (*e.g.,* toxicity, inflammation, pain, development, oncogenesis, apoptosis, etc.) both *in vivo* and *in vitro*. Using this unique approach, termed *in vivo* differential display (IVDD), gene expression in living animals can be readily studied. IVDD has many uses, including, but certainly not limited to, drug testing and development and toxicological testing for chemicals.
>
> During virtually all non-normal physiological states, organisms activate (induce) specific **genes** or groups of **genes**. Thus, infectious agents, pathological conditions, environment and/or toxic stimuli may induce the expression of certain **genes** associated with a particular biochemical pathway of physiological condition.[7] ...
>
> The control elements of the **genes** of interest are operably linked to reporter **genes** to create chimeric **genes** that [are] used to generate transgenic animals (*e.g.,* mice). These transgenic animals can then serve as test animals for example, for toxicology or stress testing. Induction of expression of these **genes** can be evaluated using non-invasive imaging.[8]

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[5] *See, e.g.,* page 2, line 29 to page 3, line 12 of the application

[6] *See, e.g.,* page 60, line 29 to page 61, line 6 of the application

[7] Page 22, lines 3-12 of as-filed specification, emphasis added to show multiple reporter constructs employed

[8] Page 23, lines 22-25 of as filed specification, emphasis added to show multiple reporter constructs employed

In view of this clear description of the claimed subject matter and the axiomatic rule that it is **not** required that animals comprising multiple reporter constructs be exemplified in order to satisfy the written description requirement, it is plain that the disclosure as a whole, including the well-known and the new, amply establishes that the written description requirement in the case on appeal has been satisfied.

Appellants have shown possession of animals and methods that provide an *in vivo* screening method using multiple reporter constructs, and, accordingly, the written description requirement of 35 U.S.C. § 112, first paragraph has been satisfied.

3. The As-Filed Specification Enables the Subject Matter of Appealed Claims 38, 40, 41, 45, 46, 49 and 65-68

Claims 38, 40, 41, 45, 46, 49 and 65-68 also remain rejected under 35 U.S.C. § 112 as allegedly not enabled by the as-filed specification. (Advisory Action, page 3). In support of this rejection, the Advisory Action states:

> ...the existence of transgenic mice expressing light generating proteins is not sufficient to enable the claimed invention because the **claimed** invention requires the generation of transgenic mice having promoters that regulate expression of the light generating proteins in a manner that is **predictive** of native gene expression (the only asserted utility for the claimed transgenic mouse). ... The specification fails to enable the identification of such promoter portions that the native context is retained when the promoter is truncated and inserted into an expression cassette, which is then inserted into the genome of a mouse (in a new genetic context).[9]

The rejection is based on multiple unsupportable assumptions.

First, the assumption that reporter constructs containing promoters of known genes operably linked to reporter molecules are not predictive of native gene expression is in error. Reporter molecules as claimed were known to predict native gene expression, both *in vivo* and *in vivo*:

---

[9] Advisory Action, page 3, emphasis in original

> During virtually all non-normal physiological states, organisms activate (induce) specific genes or groups of genes.[10]
>
> In one aspect of the invention, reporter gene expression cassettes are constructed using control elements selected from a gene or group of genes whose expression is known to be associated with a particular biochemical pathway or physiological function. For example, the control elements may be stress-inducible control elements selected from a gene or group of genes associated with cellular stress or toxicity. **Thus, toxicity can be monitored *in vivo* by analyzing expression of the reporter gene**. .... [T]he regulatory (or control) sequences confer the responsiveness to the construct, i.e., the promoter taken as a whole functions like a promoter derived from a selected gene.[11]

Second, the allegation that the specification is required to "enable" identification of promoter portions is unsupported. The specification clearly teaches that many stress-related promoters were well known at the time of filing and there are pages and pages of description and references regarding these promoters and portions thereof (including, for example, CREs) that retain their ability to predict native gene expression when truncated:

> Many stress-inducible genes have been identified, sequenced and analyzed. Information about stress-related control elements is widely available. In the practice of the present invention, stress-related control elements are selected, and operably linked to a reporter gene coding sequence, which results in the generation of chimeric genes where the reporter gene coding sequence (for example, sequences encoding a light generating polypeptide such as luciferase) are subject to the regulation provided by the stress-related control elements.[12]
>
> Each gene is controlled by a unique promoter. However, genes that respond to a particular stimuli (*e.g.*, stress, infection) can contain within their promoters, a common response element (CRE) or, associated with their promoters, regulatory or control sequences involved with regulation of expression of the gene (e.g., induction or repression). ... CREs (or other regulatory or

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[10] Page 22, lines 9-10 of the application

[11] Page 33, lines 5-10 and lines 23-25 of the application, emphasis added

[12] Page 35, lines 12-17 of the application; *see, also,* pages 35 to 41 of the application and references cited therein regarding stress-inducible promoters and portions thereof

> control elements associated with a selected gene) can be isolated [citations omitted] and operably linked to a minimal promoter and a reporter gene.[13]

Third, contrary to the Examiner's allegations, the specification teaches how to identify promoters and functional portions thereof from any gene:

> Genes, and promoters from these genes, that are induced by the aforementioned stimuli can be identified as described herein. For example, subtractive hybridization can be used to determine which transcripts are activated (or overexpressed) when the cells or animals are exposed to the stimuli of interest.[14]

Fourth, not only does the specification teach both that stress-inducible promoters (and portions thereof) were known at the time of filing and how to identify additional promoters (and regions thereof), the specification clearly defines the claim term "operably linked" such that the promoter of the reporter construct is required to performs its usual (native) function, *i.e.*, recapitulate native gene expression:

> "Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter that is operably linked to a coding sequence (e.g., a reporter expression cassette) is capable of effecting the expression of the coding sequence when the proper enzymes are present.[15]

The evidence of record clearly establishes that recapitulating native gene expression in a transgenic mouse comprising a single reporter construct (including a light-generating reporter) was known. Thus, the skilled artisan would expect that multiple of such constructs would also function as claimed, in view of the disclosure in the as-filed specification.

As set forth in the seminal case of *In re Marzocchi,* 439 F.2d, 220, 223, 169 USPQ 367, 369 (CCPA 1971), a patent application is presumptively enabled when filed:

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[13] Page 33, lines 14-25 of the application

[14] Page 22, lines 13-16 of the application

[15] *See,* page 12, line 30 to page 13, line 1 of the application

> [a]s a matter of Patent Office practice ... a specification .. must be taken as in compliance with the enablement requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

Moreover,

> it is incumbent upon the Patent Office, whenever a rejection on [grounds of enablement] is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure.

439 F.2d at 224, 169 USPQ at 369-370. Indeed, as pointed in the Patent Office's own Training Manual on Enablement (1993, citing *In re Wright,* 999 F.2d 1557, 1561-1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993), "the case law makes clear that properly reasoned and supported statements explaining any failure to comply with section 112 are a requirement to support a rejection."

In the case on appeal, the Examiner has not properly set forth why the teachings of the specification do not enable the skilled artisan to make and use the claimed subject matter. The specification establishes that single reporter constructs introduced to make transgenic animals can recapitulate native gene expression *in vivo.* The Office has not provided any reason to doubt that the multiple reporter constructs are operative.

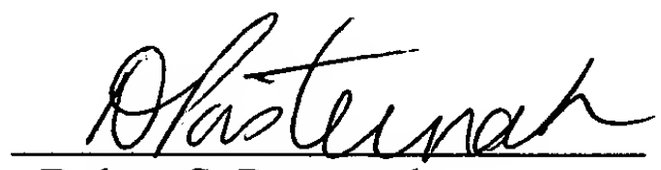
The Office has not met its burden of overcoming the evidence of record which demonstrates that the specification enables the pending claims throughout their scope. As such, the rejection cannot stand.

CONCLUSION

For the reasons stated above, Appellant respectfully submits that the specification adequately describes the pending claims and that the pending claims are patentable over the art cited by the Examiner. Accordingly, Appellant requests that the rejections of the claims on appeal be reversed, and that the application be remanded to the Examiner so that the appealed claims can proceed to allowance.

Respectfully submitted,

Date: October 19, 2006

By: 

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CLAIMS APPENDIX

CLAIMS ON APPEAL

1-37. (canceled)

38. (previously presented): A transgenic mouse comprising a panel of expression cassettes, said transgenic mouse produced by a method comprising the steps of

introducing a first expression cassette comprising a first promoter derived from a first stress-inducible gene into a mouse at an embryonic stage, said promoter operably linked to sequences encoding a first light generating polypeptide, and

introducing a second expression cassette comprising a second promoter derived from a second stress-inducible gene into said mouse at an embryonic stage, said promoter operably linked to sequences encoding a second light generating polypeptide and said second promoter derived from a different stress-inducible gene than said first promoter.

39. (canceled)

40. (previously presented): A method of determining the effect of an analyte on gene expression mediated by promoters derived from stress-inducible genes, wherein said expression is in a living transgenic mouse, said method comprising

administering the analyte to a living transgenic mouse of claim 38, wherein administering of said analyte is carried out under conditions that permit light generation mediated by said light generating polypeptide in the transgenic mouse,

determining the effect of the analyte on expression of the light generating polypeptide in a living transgenic mouse wherein said expression is mediated by at least one of the promoters.

41. (previously presented): The method of claim 40, wherein said conditions that permit light generation mediated by the light generating polypeptide includes administering, to the transgenic mouse, at least one substrate for the light generating polypeptide.

42-44. (canceled)

45. (previously presented): A noninvasive method for detecting a level of expression in response to an analyte, wherein said expression is (i) mediated by promoters derived from stress-inducible genes, and (ii) in a living transgenic mouse, said method comprising

(a) administering the analyte to a living transgenic mouse of claim 38, wherein administering of said analyte is carried out under conditions that permit light generation mediated by said light generating polypeptide,

(b) placing the transgenic mouse within a detection field of a photo detector device,

(c) maintaining the transgenic mouse in the detection field of the device, and

(d) during said maintaining, measuring photon emission from the transgenic mouse with the photo detector device to detect the level of expression of the light generating polypeptide in the living transgenic mouse wherein said expression is mediated by at least one of the promoters.

46. (previously presented): The method of claim 45, further comprising,
(e) repeating steps (b) through (d) at selected intervals, wherein said repeating is effective to detect changes in the level of the light emission in the transgenic mouse over time.

47-48. (canceled)

49. (previously presented): A method of providing a transgenic mouse suitable for screening a selected analyte, comprising

generating a transgenic mouse of claim 38, and

providing said transgenic mouse or progeny thereof for use in screening a selected analyte.

50-64. (canceled)

65. (previously presented): The transgenic mouse of claim 38, wherein the method further comprises

introducing a third expression cassette comprising a promoter derived from a third stress-inducible gene into a mouse at an embryonic stage, said third promoter operably linked to sequences encoding a third light generating polypeptide and said third promoter derived from a different stress-inducible gene than said first and second promoters.

66. (previously presented): The transgenic mouse of claim 65, wherein (i) said first, second, and third promoters are each derived from a different gene, and (ii) said first, second, and third light generating polypeptides produce the same color of light.

67. (previously presented): The transgenic mouse of claim 65, wherein (i) said first, second, and third promoters are each derived from a different gene, and (ii) at least two of said first, second, and third light generating polypeptides produce different colors of light.

68. (previously presented): The transgenic mouse of claim 65, said panel further comprising additional expression cassettes, wherein each expression cassette comprises a promoter derived from a different stress-inducible gene, said promoter operably linked to sequences encoding a light generating polypeptide.

69-80. (canceled)

EVIDENCE APPENDIX

No documents are attached to this Evidence Appendix.

RELATED PROCEEDINGS APPENDIX

As noted above on page 2 of this Brief on Appeal Appellants are not aware of any related proceedings. In as much as no decisions have been rendered by a court or the Board in this related case, no documents are submitted with the Evidence Appendix.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Alexandria, VA 22313 on **October 19, 2006**.

10/19/06 Michelle Hobson

Date Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: ZHANG et al. Serial No.: 09/464,795 Filing Date: December 16, 1999 Title: NON-INVASIVE EVALUATION OF PHYSIOLOGICAL RESPONSE IN A MAMMAL	Examiner: Anne Marie Falk Group Art Unit: 1632 Confirmation No.: 8087 Customer No.: 20855

REPLY BRIEF

Mail Stop Appeal Brief
Commissioner for Patents
Alexandria, VA 22313

Sir:

Appellant submits one copy of this reply brief on appeal in accordance with 37 C.F.R. § 41.41. An Examiner's Answer was mailed on August 24, 2006, making a Reply Brief due on or before October 24, 2006. Accordingly, this Reply Brief is timely filed. Applicants note that a Notice of Non-Compliant Appeal Brief was mailed after the mailing of the Examiner's Answer. A revised Appeal Brief is being filed concurrently with this Reply Brief to correct improper references throughout the Brief to claim 43 as on appeal rather than canceled. Thus, because no substantive changes were made to the Appeal Brief, Applicants Reply Brief addresses the issues raised in the Examiner's Answer.

STATUS OF THE CLAIMS

Claims 38, 40, 41, 45, 46, 49 and 65-68 are currently pending in the above-referenced case (hereinafter "the application") as shown in the Claims Appendix. Claims 1-37, 39, 42, 43, 44, 50-64 and 69-80 were canceled. Therefore, pending claims 38, 40, 41, 45, 46, 49 and 65-68 are appealed and stand rejected under 35 U.S.C. § 101 and 35 U.S.C. § 112, 1st paragraph (written description and enablement).

GROUNDS OF REJECTION

1. Claims 38, 40, 41, 45, 46, 49 and 65-68 stand rejected under 35 U.S.C. § 101 as allegedly lacking patentable utility.

2. Claims 38, 40, 41, 45, 46, 49 and 65-68 stand rejected under 35 U.S.C. § 112, 1st paragraph as allegedly not adequately described by the specification as filed.

3. Claims 38, 40, 41, 45, 46, 49 and 65-68 stand rejected under 35 U.S.C. § 112, 1st paragraph as allegedly not enabled by the specification as filed.

ARGUMENTS

1. The Examiner's Answer Relied on Newly Cited References

The Examiner cited new references in the Examiner's Answer. Pursuant to 37 C.F.R. § 41.39, Appellants request that the appeal be maintained.

2. The Appealed Claims Have Patentable Utility

Claims 38, 40, 41, 45, 46, 49 and 65-68 remain rejected under 35 U.S.C. § 101 as allegedly lacking patentable utility. (Examiner's Answer, pages 3-7).

a. The Rejection

The rejection is again based on the following premises:

(1) that a "promoter derived from a stress-inducible element" includes too many embodiments that may not recapitulate gene expression to be operably and/or useful; (Examiner's Answer, pages 3-7)

(2) that the only asserted utility for transgenic mice as claimed is to "recapitulate native gene expression" (Examiner's Answer, pages 3-7);

(3) the references cited in background section of the specification regarding use of reporter genes *in vitro* and *in vivo* and to making transgenic mice are not of record (Examiner's Answer pages 18 and 19);

(4) these references do not pertain to double, triple or other multtransgenic mice (Examiner's Answer, page 18);

(5) the regulatory regions of these references were not the focus of study and therefore it cannot be said that the regulatory region provide for **native** gene expression (Examiner's Answer, page 19);

(6) the claims encompass species that lack utility (citing M.P.E.P. § 2107.02(I)) (Examiner's Answer, page 19); and

(7) there is no "evidence of record" establishing that promoter portions would recapitulate native gene expression.

b. A "promoter derived from a stress-inducible gene" necessarily recapitulates native expression of a stress-inducible gene

With regard to the point (1), Appellants again note that the Examiner's assertion that there are "promoters derived from stress-inducible genes" that do not act as transcriptional control elements when used in reporter constructs in the same way as they would *in vivo* for stress-inducible genes is contrary to the specification and common knowledge at the time of filing, as evidenced by the specification, references of record and declaratory evidence of record.

As noted above, the Examiner's Answer asserts that the claimed expression vectors cover "minimal promoters, truncated promoters and promoters lacking their endogenous enhancers and inducible elements ... [and] must necessarily be truncated" for insertion into the expression vector. (Examiner's Answer, pages 3-4). It is maintained that mice containing such "non-native" promoter sequences could not be used for the asserted utility of recapitulating native gene expression.

As clearly used throughout the specification, the term "promoter derived from a stress-inducible gene" is a sequence that controls transcription of a gene to which it is operably linked in the same way it would control transcription of a stress-inducible gene. The terms "promoter" and "control element" are used interchangeably (page 34, lines 13-31, emphasis added):

> Each gene is controlled by a unique **promoter**. However, genes that respond to a particular stimuli (*e.g.*, stress, infection) can contain within their promoters, a common response element (CRE) or, associated with their promoters, regulatory or control sequences involved with regulation of expression of the gene (e.g., induction or repression). ... CREs (**or other regulatory or control elements associated with a selected gene**) can be isolated [citations omitted] and operably linked to a minimal promoter and a reporter gene.
>
> **The control element (*e.g.*, promoter)** may be from the same species as the transgenic animal ..... The control element can be derived from any gene of interest by methods known in the art.

Moreover, the term "promoter derived from a stress inducible gene" refers only to sequences that mimic the native control expression of a stress-inducible gene (*see, e.g.*, page 35, lines 11-17, emphasis added):

> Many stress-inducible genes have been identified, sequenced, and analyzed. Information about stress-related control elements is widely available. In the practice of the present invention, stress-related control elements are selected, and operably linked to a reporter gene, which results in the generation of chimeric genes where the reporter gene coding sequences (for example, sequences encoding a light-generating polypeptide such as luciferase) are subject to the regulation provided by the stress-related control elements.

See, also, page 33, line 30 to page 34, line 7, noting that:

> Before use of the expression cassette to generate a transgenic animal, the responsiveness of the expression cassette to the stress-inducer associated with selected control elements can be tested by introducing the expression cassette into a suitable cell line (*e.g.*, primary cells, transformed cells, or immortalized cell lines).
>
> The control elements of the genes of interest are operably linked to reporter genes to create chimeric genes (*e.g.*, reporter expression cassettes) that are used to generate transgenic animals (for example, mice). These transgenic animals can then serve as test animals as described herein. Induction, repression, or any state of expression of such reporter expression cassette genes can be evaluated using non-invasive imaging.

Furthermore, the alleged "combination" of control elements does not change the fact that claim term "promoter derived from a stress-inducible gene" only includes those sequences that regulate expression of the light-generating polypeptide in the same way as they regulate the gene from which they are derived.

In addition to the fact that the specification clearly teaches that the promoter derived from a stress-inducible gene must recapitulate native gene expression by mimicking the transcriptional effects of any stress-inducible control element, Appellants believe that the Examiner's interpretation of the term "promoter derived from a stress-inducible gene" to include any sequence that drives expression of a coding sequence when operably linked thereto, fails to comport with the knowledge of one of skill in the art in the field of molecular biology.

As previously noted, it was well known at the time of filing how to identify promoter elements from any gene of interest. Promoters from inducible genes would clearly themselves mimic that inducibility – otherwise, they were would not be promoters derived from a stress-inducible gene, but enhancers, constitutive promoters or the like. Thus, promoters derived from a stress-inducible gene do not include sequences which drive expression of any gene. Only those sequences which control expression of a stress-inducible gene are encompassed by the definition.

Nor does is a promoter sequence that recapitulates transcriptional control exerted over a stress-inducible gene in nature required to have "all the relevant native regulatory regions" in

order to modulate promoter function. To assert that the term "promoter derived from a stress-inducible gene" includes sequences that do not recapitulate native gene expression when linked to a reporter gene stretches the meaning of the term beyond credulity. The skilled artisan would clearly recognize that the "promoter derived from a stress-inducible gene" is a sequence that, by definition, control expression of the gene to which it is operably linked only in the same way that it would control expression of it natural gene (a stress-inducible gene).

In light of the art, as exemplified by the references discussed above, the term "promoter derived from a stress-inducible gene" cannot be construed to encompass sequences that do not include all native regulatory regions and/or sequences that do not drive expression of a stress-inducible gene. Indeed, the importance of construing claim language in light of the art was recently reaffirmed by the Federal Circuit, *en banc*, in *Phillips v. AWH*, 415 F.3d 1303, 75 USPQ2d 1321 (Fed. Cir. 2005). Therein, the court, citing a number of previous decisions,[1] confirmed its precedent that claim terms are given their ordinary and customary meaning to a person of ordinary skill in the art at the effective filing date of the patent application:

> We have made clear, moreover, that the ordinary and customary meaning of a claim term is the meaning that the term would have to a person of ordinary skill in the art in question at the time of the invention, i.e., as of the effective filing date of the patent application.[2]

The effective filing date of the application providing support for the claimed subject matter is December 16, 1999. As the skilled artisan was well aware at that date, a "promoter derived from a stress-inducible gene" would necessarily be inducible by the same molecules (stressors) that induce the stress-inducible gene in nature. Thus, the meaning attributed to the term "promoter derived from a stress-inducible gene" by the Examiner is not the meaning of that term as set forth in the specification or the meaning of the term to one of skill in the relevant art.

---

[1] See, for example, *Vitronics Corp. v. Conceptronic, Inc.* 90 F.3d 1576, 1582 (Fed. Cir. 1996); *Ferguson Beauregard/Logic Controls v. Mega Sys., LLC*, 350 F.3d 1327, 1338 (Fed. Cir. 2003) and *Home Diagnostics, Inc. v. LifeScan, Inc.*, 381 F.3d 1352, 1358 (Fed. Cir. 2004)

[2] *Phillips v. AWH Corp.*, 75 USPQ2d 1321, 1326 (Fed. Cir. 2005)

Thus, the law, including *Phillips*, plainly establishes that the Examiner's interpretation of the claims on appeal is incorrect.

c. The Asserted Utility

Based on the erroneous interpretation of the term "a promoter derived from a stress-inducible gene," the Examiner continues to assert that the "vast majority" of the claimed "artificial" constructs do not have the asserted utility, essentially because they lack all relevant **native** regulatory regions. (Examiner's Answer, page 5).

The asserted utility is using live mice containing reporter constructs to monitor the effect of any molecule on the promoter. As noted above and throughout prosecution, there are innumerable references establishing that reporter constructs containing a promoter derived from a selected gene operably linked to a reporter gene **recapitulate gene expression** such that the effect of a molecule on that gene (via the promoter) can be determined (see, page 3, lines 2-33 of the application; *see, also;* page 22, lines 2-12 of the application:

> Cardiovascular biology and diseases have been investigated in Tg mouse models using tissue-specific promoters [citations omitted] and regulation of insulin-responsive glucose transporter GLUT4 and Apo A-I genes have also been studied in models of diabetes, obesity [citation omitted] and coronary artery disease [citations omitted].
>
> Photoproteins as biological labels have been used for more than a decade for the study of gene expression in cell culture or using excised tissue [citations omitted]. Low-light imaging of internal bioluminescent signals has been used to study temporal and spatial gene regulation in relatively thin or nearly transparent organisms [citations omitted]. External detection of internal light penetrating the opaque tissues has been described [citation omitted].

Preparing a transgenic mouse comprising a reporter construct as claimed (inducible promoter operably linked to light-generating reporter) is in fact well-described and claimed in these references, including references which are of record (see, *e.g.,* Evidence Appendices (1) and (2), U.S. Patent Nos. 5,650,135 and 6,217,847, references AA-1 and AA-3 of IDS submitted on August 21, 2001, for example claim 1 and col. 3, lines 15-27 of U.S. Patent No. 6,217,847):

1. A method for detecting a promoter-induction event in an animal, said method comprising the steps:

(a) triggering the event in a transgenic animal having a promoter responsive to such event and a heterologous gene encoding a light-generating protein under control thereof, and

(b) measuring with a photodetector device, photon emission through opaque tissue from expressed light-generating protein in the animal.

...

In a related embodiment, the invention includes a noninvasive method for detecting the localization of a promoter-induction event in an animal made transgenic or chimeric for a construct including a gene encoding a light-generating protein under the control of an inducible promoter. Promoter induction events include the administration of a substance which directly activates the promoter, the administration of a substance which stimulates production of an endogenous promoter activator (e.g., stimulation of interferon production by RNA virus infection), the imposition of conditions resulting in the production of an endogenous promoter activator (e.g., heat shock or stress), and the like. The event is triggered, and the animal is imaged as described above.

As with the pending claims, the entire promoter does not need to be used and methods of identifying inducible promoters for use in transgenic reporter construct-containing animals was well established (*see,* U.S. Patent No. 6,217,847, col. 15, lines 1-41):

D. Transgenic Animals Containing Genes Encoding Light-Generating Proteins

In another aspect, the present invention includes transgenic animals containing a heterologous gene construct encoding a light-generating protein or complex of proteins. The construct is driven by a selected promoter, and can include, for example, various accessory proteins required for the functional expression of the light-generating protein, as well as selection markers and enhancer elements.

Activation of the promoter results in increased expression of the genes encoding the light-generating molecules and accessory proteins. Activation of the promoter is achieved by the interaction of a selected biocompatible entity, or parts

of the entity, with the promoter elements. If the activation occurs only in a part of the animal, only cells in that part will express the light-generating protein. ...

It is also possible to use methods of the invention with tissue-specific promoters. This enables, for example, the screening of compounds which are effective to inhibit pathogenic processes resulting in the degeneration of a particular organ or tissue in the body, and permits the tracking of cells (e.g., neurons) in, for example, a developing animal.

Many promoters which are applicable for use with the present invention are known in the art. In addition, methods are known for isolating promoters of cloned genes, using information from the gene's cDNA to isolate promoter-containing genomic DNA.

Thus, contrary to the Examiner's assertion, the references relating to transgenic mice comprising a single reporter construct including only portions of a selected promoter as claimed are of record.

More importantly, the references of record, for example, as reproduced above, completely rebut the assertion that "regulatory regions included in those transgenes were not the focus of the study." *See, e.g.,* U.S. Patent Nos. 5,650,135 and 6,217,847, reproduced above.

Even if these references were not of record, it is clear that making transgenic animals comprising reporter constructs as claimed was standard at the time of filing. Simply put, the utility of using reporter constructs *in vivo* in transgenic animals to screen for promoter effects (using truncated promoters) is a well-established utility and, on this basis alone, the utility rejection cannot stand.

d. The Claims Do Not Encompass Species which Lack the Asserted Utility

The Examiner also errs in asserting that the claims encompass "species which lack utility." Every transgenic mouse that includes at least one reporter construct that includes a promoter derived from a stress-inducible gene operably linked to a light-generating promoter has the asserted and well-established utility of determining a promoter-induction event. *See, also,* U.S. Patent Nos. 5,650,135 and 6,217,847, Evidence Appendices (1) and (2), reproduced in part

above. Consequently, the claims do not encompass species lacking utility and M.P.E.P. § 2107.02(I) is not relevant.

e. The Evidence of Record

It is also error to assert that there is no evidence that promoter portions as claimed would recapitulate native gene expression. The evidence relied on includes the specification as-filed, Declaratory evidence of record, of-record references, and the state of the art (*e.g.,* as discussed in the specification). This evidence does not need to be included in the Evidence Appendix of an Appeal Brief in order to be considered by the Board.

Nonetheless, Applicants have included in the Evidence Appendix attached hereto, copies of U.S. Patent Nos. 5,650,135 and 6,217,847 as well as the Declaration of David West. Along with the specification, this constitutes much of the "evidence of record" relied on by Appellants.

3. The As-Filed Specification Describes the Subject Matter of the Appealed Claims

a. The Rejection

Claims 38, 40, 41, 45, 46, 49 and 65-68 also remain rejected under 35 U.S.C. § 112 as allegedly not adequately described by the as-filed specification. (Examiner's Answer, pages 7-11). In support of this rejection, the Examiner's states that:

(1) the specification does not provide any working example for producing a transgenic mouse as claimed and does not describe the expression characteristics of a transgenic mouse encompassed by the claimed invention (Examiner's Answer, page 8);

(2) due to alleged unpredictability of site of integration of the transgene, the final genetic context of the expression cassette is not described (*Id.*);

(3) the specification does not describe "which portions of the promoter are to be included" (Examiner's Answer, page 9);

(4) "stress-inducible elements are not required to be present in the expression cassettes" (*Id.*); and

(5) the specification does not describe "those regulatory regions that are critical, where they are located or their upstream boundaries" (Examiner's Answer, page 20).

Essentially, as with the utility rejection, the written description rejection rests on the assertion that the specification has not adequately described the promoters and/or transgenic animals comprising constructs that provide for **native** gene expression. (Examiner's Answer, pages 9-10). Cameron and Wood are cited for allegedly teaching that transgenic animals as claimed cannot be adequately described because of the effect of integration on expression and the unpredictability of the phenotype of a transgenic mouse. (Examiner's Answer, pages 10-11).

b. The Definition of a "Promoter Derived From a Stress-Inducible Gene"

Appellants again submit, for the reasons of record and noted above, that the Examiner's definition of the term "promoter derived from a stress-inducible gene" to encompass sequences that do not mimic their native promoter activity is untenable.

In both common usage and as used throughout the as-filed specification, the term "promoter derived from a stress-inducible gene" includes only those sequences that regulate expression of the reporter gene to which it is operably linked in the same manner as it would regulate the gene to which it is operably linked in nature. *See,* Section 2.b. of this Reply Brief above. In other words, a promoter derived from a stress-inducible gene will control expression of the reporter gene in the same way it controls expression of the native stress-inducible gene.

Furthermore, as noted above, promoters from stress-inducible genes were known and, hence described, at the time of filing (*see, e.g.,* page 35, lines 12-13 of the as-filed specification stating that "[m]any stress-inducible genes have been identified, sequenced, and analyzed. Information about stress-related control elements is widely available.") As detailed in the Appeal Brief (citing, *e.g., Capon v. Eshhar*) and reiterated below (*see, e.g., Falkner v. Inglis*) re-description of known biomolecules (promoters) is never required in order to have adequate written description.

c. Working Examples Are Not Required to Show Adequate Written Description

It is axiomatic that the adequacy of a specification's written description is not determined by what is exemplified and, in fact, the Federal Circuit, the Board, the M.P.E.P. and the PTO's own Training Materials forbid such a test.[3] Thus, the written description requirement of 35 U.S.C. § 112, first paragraph should not be used to reject every broad, pioneering invention. Instead, each application must be judged on the particular fact pattern (disclosure, state of the art, *etc.*) with the underlying assumption that the specification as filed is presumed to satisfy the written description requirement. *See, e.g., In re Wertheim,* 541 F.2d 257, 265, 191 USPQ 90, 98 (CCPA 1976).

Recently, in *Falkner v. Inglis,* 79 USPQ2d 1001 (Fed. Cir. 2006), the Federal Circuit reaffirmed that working examples are not required to satisfy the written description requirement, even for a broad genus (*see, Falkner,* page 1007):

> Specifically, we hold, in accordance with our prior case law, that (1) examples are not necessary to support the adequacy of a written description (2) the written description standard may be met (as it is here) even where actual reduction to practice of an invention is absent; and (3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure.

Thus, satisfaction of the written description requirement in the case on appeal does not require a working example of a transgenic animal containing two or more expression cassettes as claimed.

d. Promoter Elements derived from Stress-inducible Genes were known and are described

The Federal Circuit's holding in *Falkner* is also particularly relevant to the Examiner's assertion that the specification does not adequately describe the promoters of the claimed

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[3] Indeed, as the Examples of PTO Training Materials on Written Description, including Example 14: "Product by Function," make clear, disclosure of a single species can readily satisfy the written description requirement for broad claims.

constructs. In *Falkner,* the Federal Circuit cited *Capon* in reaffirming that adequate written description does not require re-description of the sequence of known molecules and that literature available at the time of filing must be considered in determining the adequacy of the written description (*Falkner*, page 1007):

> Indeed, a requirement that patentees recite known DNA structures, if one existed, would serve no goal of the written description requirement. It would neither enforce the quid pro quo between the patentee and the public by forcing the disclosure of new information, nor would it be necessary to demonstrate to a person of ordinary skill in the art that the patentee was in possession of the claimed invention. As we stated in Capon, "[t]he 'written description' requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution." Id. at 1358. Indeed, the forced recitation of known sequences in patent disclosures would only add unnecessary bulk to the specification. Accordingly we hold that where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here "essential genes"), satisfaction of the written description requirement does not require either the recitation or incorporation by reference (wherein permitted) of such genes and sequences.

The holding in *Falkner* provides further support (if any is needed) that the written description rejection in the case on appeal is unsustainable in view of the specification as a whole (*e.g.,* the teachings that promoters from stress-inducible genes were well known and analyzed, *see,* page 35, lines 10-15) and the state of the art as exemplified by the literature of record (*e.g.,* U.S. Patent Nos. 5,650,135 and 6,217,847, establishing that reporter constructs as claimed could be made with any promoter).

In light of these clear teachings of the court, the Office's assertion, in the case on appeal, that Appellants are required to disclose multiple examples (representative species) of particular promoter constructs and/or animals containing these constructs, is inconsistent with the requirements of the first paragraph of Section 112.

In the case on appeal, the state of the art and as-filed specification clearly establish that transgenic animals comprising reporter constructs as claimed can be used to evaluate promoter induction events, even when a "truncated promoter" is used. *See, e.g.,* U.S. Patent No. 6,217,847, which is related to U.S. Patent No. 6,530,135, cited below as found in the as-filed specification (*see,* page 2, line 29 to page 3, line 12 and page 60, line 29 to page 61, line 6 of the as-filed specification):

> A wide range of Tg mice that employ reporter constructs have been developed and tested. For example, Tg mice containing the viral long terminal repeat (LTR) promoter fusions have been used to study the range of tissues and cells types that are capable of supporting HTLV-1 expression and the development of neurofibromatosis-like tumors associated with HTLV-1 retrovirus ... [citations omitted]. Cardiovascular biology and diseases have been investigated in Tg mouse models using tissue-specific promoters [citations omitted], and regulation of insulin-responsive glucose transporter GLUT4 and Apo A-I genes have also been studied in models of diabetes, obesity [citation omitted] and coronary artery disease [citation omitted].
>
> Photoproteins as biological labels have been used for more than a decade for the study of gene expression in cell culture or using excised tissue... ...
>
> The monitoring of *lux/luc* reporter genes using non-invasive whole animal imaging has been described [citing U.S. Patent No. 5,650,135 and other citations].

As noted repeatedly, U.S. Patent No. 5,650,135 and related U.S. Patent No. 6,217,847 describes transgenic animals in which promoter induction events are monitored by evaluating expression of a reporter gene operably linked to a promoter sequence. The promoter sequence does not have to be full length to mimic its native promoter activity.

Thus, Appellants have shown possession of animals and methods that provide an *in vivo* screening method using multiple reporter constructs, and, accordingly, the written description requirement of 35 U.S.C. § 112, first paragraph has been satisfied.

e. The References Do Not Establish "Unpredictability" and Are Not Relevant to Written Description

Predictability or alleged unpredictability is not a particularly relevant factor in a written description inquiry. Thus, the teachings of Cameron and Wood regarding context of integration is not germane to the adequacy of written description in the case on appeal.

In any event, Cameron and Wood relate to insertion (or knock out) of a gene that encodes a protein of interest, not a reporter gene as claimed. Accordingly, issues raised in these references regarding the context of integration, leaky expression, the influence of copy number, issues do not relate to reporter constructs specifically, as claimed. Likewise, the complex interaction of genes that results in a particular phenotype as described in Wood, is not relevant to claims directed to transgenic animals comprising a reporter construct as claimed. Unlike Cameron's and Wood's animals, it does not matter where the construct integrates in the claimed animals because the phenotype of the animal is changed only with respect to light emitted in response to activation of the stress-inducible promoter.

Thus, even if satisfaction of the written description requirement necessitated a showing by Appellants regarding "predictability" (which it does not), Cameron and Wood are not germane to the instant invention.

4. The As-Filed Specification Enables the Subject Matter of the Appealed Claims

a. The Rejection

Claims 38, 40, 41, 45, 46, 49 and 65-68 also remain rejected under 35 U.S.C. § 112 as allegedly not enabled by the as-filed specification, for the reasons of record and in view of various newly cited references allegedly established unpredictability. (Examiner's Answer, pages 12-18). In response to Appellants' arguments in the Appeal Brief, the Examiner stated:

(1) no support was offered for the assertion that reporter molecules as claimed were known to predict native gene expression (Examiner's Answer, page 20);

(2) the specification fails to enable identification of promoter "portions" necessary and sufficient to achieve native patterns of gene expression (Examiner's Answer, pages 20-21);

(3) the specification does not provide guidance for creating constructs that recapitulate native gene expression (Examiner's Answer, page 21); and

(4) there is no "evidence of record" establishing that promoter portions would recapitulate native gene expression (Examiner's Answer, page 22).

Because the specification teaches how to make and use the claimed transgenic animals, Appellants traverse the rejection and supporting remarks.

The test of enablement is whether one of ordinary skill in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *Ex parte Forman*, 230 USPQ 546 (BPAI 1986). *See, also,* M.P.E.P. § 2164.01 which states the test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art, citing *United States v. Telectronics Inc.,* 8 USPQ2d 1217 (Fed. Cir. 1988), *cert. denied,* 490 U.S. 1046 (1989).

As set forth in the seminal case of *In re Marzocchi,* 439 F.2d, 220, 223, 169 USPQ 367, 369 (CCPA 1971), a patent application is presumptively enabled when filed:

> [a]s a matter of Patent Office practice ... a specification .. must be taken as in compliance with the enablement requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

Moreover,

> it is incumbent upon the Patent Office, whenever a rejection on [grounds of enablement] is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure.

439 F.2d at 224, 169 USPQ at 369-370. Indeed, as pointed in the Patent Office's own Training Manual on Enablement (1993, citing *In re Wright,* 999 F.2d 1557, 1561-1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993), "the case law makes clear that properly reasoned and supported

statements explaining any failure to comply with section 112 are a requirement to support a rejection."

The pending claims are drawn to transgenic animals comprising two reporter constructs. Each reporter construct comprises a promoter derived from a stress-inducible gene operably linked to a sequence encoding a light-generating protein.

The Examiner has not set forth why the teachings of the specification do not enable one of skill in the art to make and/or use such transgenic animals. Indeed, as with written description, the enablement rejection is based on the erroneous interpretation of the term "promoter derived from a stress-inducible gene"; the erroneous assertion that various references somehow establish unpredictability; and the erroneous assertion that only that which is exemplified is enabled.

b. The Specification Enables Construction of Reporter Molecules that Recapitulate Native Gene Expression, including minimal or truncated promoter elements

Contrary to the Examiner's assertions, there is ample guidance on how to make reporter constructs as claimed that were known to recapitulate gene expression, both *in vitro* and *in vivo* and which reporter constructs include less than the full-length promoter, for example "minimal" or "selected" promoter elements. This guidance is found in all the evidence of record – as-filed specification, of-record references and declaratory evidence.

As noted in the Background Section of the specification, it was completely routine at t he time of filing to recapitulate native gene expression *in vitro* using light emitting reporter genes operably linked to inducible promoters. *See,* page 3, lines 11 to 16, emphasis added: "Photoproteins as biological **labels have been used for more than a decade for the study of gene expression** in cell culture or using excised tissue [citations omitted].

Similarly, with regard to *in vivo* systems, Appellants have provided ample evidence that reporter molecules as claimed recapitulate gene expression. This evidence includes the as filed specification (*see, e.g.,* page 2 line 29 to page 3, line 10, establishing that operably linking

inducible-promoters to reporter genes allows the evaluation of transcriptional regulation of the gene which is naturally regulated by the promoter, emphasis added):

> A wide range of Tg mice that employ reporter constructs have been developed and tested. For example, Tg mice containing the viral long terminal repeat (LTR) **promoter fusions have been used to study** the range of tissues and cells types that are capable of supporting **HTLV-1 expression** and the development of neurofibromatosis-like tumors associated with HTLV-1 retrovirus ... [citations omitted]. The LTR from HIV-1 has been fused to luciferase **to evaluate transcriptional regulation** by UV light and various sensitizing agents [citations omitted]. Cardiovascular biology and diseases have been investigated in **Tg mouse models using tissue-specific promoters** [citations omitted], and **regulation of insulin-responsive glucose transporter** GLUT4 and Apo A-I genes have also been studied in models of diabetes, obesity [citation omitted] and coronary artery disease [citation omitted].

As noted above, the specification also plainly teaches that the reporter constructs used to make the transgenic animals recapitulate native gene expression (Section 3.1.0, beginning on page 33, *e.g.,* page 33, lines 23-25; page 34, lines 20-27; page 34, line 30 to page 35, line 9, emphasis added):

> CREs (**or other regulatory or control elements associated with a selected gene**) can be isolated (for example, by producing the sequence synthetically or by polymerase chain reaction amplification from a template [citations omitted] and **operably linked to a <u>minimal</u> promoter and a reporter gene**. In this case, the regulatory (or control) sequences confer the responsiveness to the construct, i.e., **the promoter taken as a whole functions like a promoter derived from a selected gene**.
>
> Reporter expression cassettes useful in the practice of the present invention can be constructed using any control element of interest operably linked to suitable reporter gene coding sequences. .... In addition, the expression cassette can be used either directly for the generation of transgenic animals or placed in any number of vectors useful for the generation of transgenic animals. These animals (test animals) can then be used for examining *in vivo* effects of a selected analyte (for example, a drug of interest) **on expression mediated by the selected control element(s)**.

Before use of the expression cassette to generate a transgenic animal, **responsiveness of the expression cassette to the stress-inducer associated with the selected control elements** can be tested by introducing the expression cassette into a suitable cell line (e.g., primary cells, transformed cells or immortalized cell lines).

The control elements of the genes of interest are operably linked to reporter genes to create chimeric genes (e.g., reporter expression cassettes) that are used to generate transgenic animals (for example, mice). These transgenic animals can then serve as test animals as described herein. Induction, repression **or any state of expression of such reporter expression cassette genes can be evaluated** using non-invasive imaging.

Additional evidence or record, namely references of record and declaratory evidence, is in accord. As noted above, U.S. Patent Nos. 5,650,135 and 6,217,847 clearly establish that reporter constructs comprising an inducible promoter operably linked to a light-generating protein can be are used to study gene expression (*i.e.,* recapitulate native gene expression). *See,* Evidence Appendices (1) and (2) and portions of these patents reproduced above.

Furthermore, Dr. West's Declaration also establishes that reporter molecules as claimed were (and remain) the well-established gold standard for recapitulating gene expression and that construction of such reporter constructs was routine (Evidence Appendix (3), paragraphs 6-10, emphasis added):

> 6. When the specification was filed, it clearly conveyed to a typical scientist that the inventors had in their possession the invention of the claims (as set forth in paragraph 4, above). By "in their possession," I mean that the inventors contemplated transgenic mice comprising a panel of expression cassettes, wherein the panel comprises at least two different expression cassettes, each having a different stress-inducible control element operably linked to sequence encoding a light-generating polypeptide, and that they had, using the specification and information available to a typical scientist, a practical way of making and using such transgenic mice. Thus, I believe that a typical scientist would have understood the specification clearly described all of the various aspects of the claims and **enabled** a typical scientist to make and use the invention as set forth in the pending claims. I base this belief on the facts set forth below.
>
> 7. First, at the time the specification was filed, it was widely known how to construct expression cassettes generally. With regard to expression vectors comprising control elements from stress-inducible promoters operably

linked to a sequence encoding a light-generating polypeptide, such methods are described in detail in the specification, for example, in Section 3.1.0. of the specification. **Therefore, it is my opinion that construction of a panel of expression cassettes as set forth in the claims would have been routine to a typical scientist working in this area in view of the teachings of the specification**.

8. Second, it would have been clear to a typical scientist that the inventors had in their possession the various polynucleotide components of the expression cassettes. **Control elements derived from stress-inducible genes were known and clearly set forth in the specification at the time of filing**. ...

9. Third, it would have been plain to a typical scientist from the specification that the inventors were in possession of an operative way of making the claimed transgenic mice. The specification describes methods of making transgenic animals on page 59, line 28 to page 60, line 8 and in the references cited therein. **At the time the application was originally filed, such methods were routine to the typical scientist.** Indeed, methods of introducing multiple expression constructs, each with their own separate promoter, to create transgenic founders are described in the art. (See, *e.g.,* Jankowsky et al. (2001) *Biomol Eng* 17(6):157-165, copy of the Abstract attached hereto). **Also routine at the time of filing were methods of assaying if a sequence from an expression cassette had been integrated into a host mouse's genome and, if so, where such integration occurred.** Such assay methods include, but are not limited to, PCR, Northern and/or Southern blotting (for example of particular tissues) as well as *in situ* hybridization and/or imaging techniques.

10. Fourth, a typical scientist would have known that the inventors were in possession of operative methods of using these transgenic mice, for example, to determine the effect of an analyte. The evaluation of whole transgenic animals having light-reporter systems is described on line 29, page 60 through line 6, page 61 of the specification. ...

The specification and evidence of record (specification, references of record and declaratory evidence, including attached Jankowsky Abstract), establishes that the guidance provided in the specification more than amply enables the skilled artisan to make and use the claimed animals.

A *prima facie* case of non-enablement cannot be made out when the evidence of record (as-filed specification, references, declaratory evidence) clearly teaches the skilled artisan: how to select or obtain **any** promoter derived from **any** stress-inducible gene (*see,* page 33, lines 30-

31; page 35, lines 12-15; Evidence Appendices); how to construct a construct such that the promoter mimic native regulation of expression of the stress-inducible gene (Section 3.1.0 beginning on page 33, Evidence Appendices); how to create transgenic animals comprising these reporter constructs (see, e.g., Section 4.0.0, Evidence Appendixes); and how to monitor the transgenic animals for gene expression by monitoring light emitted from the reporter construct (see, e.g., Section 4.0.0, Evidence Appendices).

c. Working Examples Are Not Required

The evidence of record also belies the assertion that the claimed subject matter is not enabled because transgenic animals must actually be made experimentally. It has long been settled that only undue experimentation precludes enablement. See *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). More recently, the permissibility of routine experimentation has been reaffirmed in *Falkner v. Inglis* (cited above), wherein, in commenting on the enablement standard, the Federal Circuit stated:

> The Board observed that "the mere fact that the experimentation may have been difficult and time consuming does not mandate a conclusion that such experimentation would have been considered to be 'undue' in this art. . . ." . . . [W]e cannot say that the Board erred.

Given the evidence of record (specification, references and declaratory evidence, see Evidence Appendices), it is clear that, by following the teachings of the specification, the skilled artisan could readily the claimed animals without undue experimentation. Thus, the Office has not established a *prima facie* case of non-enablement and the rejection should be withdrawn.

d. The References Do Not Establish "Unpredictability"

The references cited in regard to the alleged "unpredictability" of transgenic animals are not relevant to the pending claims because only Cui relates to transgenic animals comprising reporter constructs as claimed. All other references relate to insertion of a non-reporter, coding sequence (*e.g.*, Hammer et al. to HLA-b27 and beta-2 microglobulin).

With regard to Cui, Appellants note that this reference was published in 1994 -- fully 5 years before the earliest priority date of the pending application. Therefore, Cui does not represent the state of the art with regard to transgenic animals comprising reporter genes at the time of filing. Indeed, by the time the instant application was filed, U.S. Patent Nos. 5,650,135 and 6,217,847 had demonstrated that very low levels of light expressed from reporter constructs can be monitored in animals.

Dr. West concurs that the specification, at the time of filing, enables the skilled artisan to make and use the claimed animals and cited Jankowski (2001) as still further evidence that the teachings of the specification provide sufficient guidance to allow the skilled artisan to make and use the claimed animals without undue experimentation.

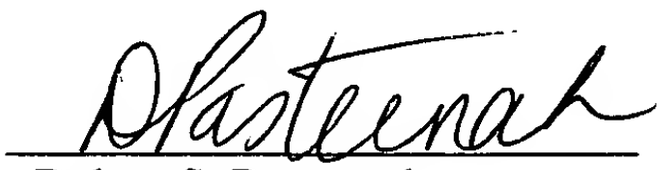
The Office has not met its burden of overcoming the evidence of record which demonstrates that the specification enables the pending claims throughout their scope. As such, the rejection cannot stand.

CONCLUSION

For the reasons stated above, Appellant respectfully submits that the specification adequately describes the pending claims and that the pending claims are patentable over the art cited by the Examiner. Accordingly, Appellant requests that the rejections of the claims on appeal be reversed, and that the application be remanded to the Examiner so that the appealed claims can proceed to allowance.

Respectfully submitted,

Date: October 19, 2006

By: 
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CLAIMS APPENDIX

CLAIMS ON APPEAL

1-37. (canceled)

38. (previously presented): A transgenic mouse comprising a panel of expression cassettes, said transgenic mouse produced by a method comprising the steps of

introducing a first expression cassette comprising a first promoter derived from a first stress-inducible gene into a mouse at an embryonic stage, said promoter operably linked to sequences encoding a first light generating polypeptide, and

introducing a second expression cassette comprising a second promoter derived from a second stress-inducible gene into said mouse at an embryonic stage, said promoter operably linked to sequences encoding a second light generating polypeptide and said second promoter derived from a different stress-inducible gene than said first promoter.

39. (canceled)

40. (previously presented): A method of determining the effect of an analyte on gene expression mediated by promoters derived from stress-inducible genes, wherein said expression is in a living transgenic mouse, said method comprising

administering the analyte to a living transgenic mouse of claim 38, wherein administering of said analyte is carried out under conditions that permit light generation mediated by said light generating polypeptide in the transgenic mouse,

determining the effect of the analyte on expression of the light generating polypeptide in a living transgenic mouse wherein said expression is mediated by at least one of the promoters.

41. (previously presented): The method of claim 40, wherein said conditions that permit light generation mediated by the light generating polypeptide includes administering, to the transgenic mouse, at least one substrate for the light generating polypeptide.

42-44. (canceled)

45. (previously presented): A noninvasive method for detecting a level of expression in response to an analyte, wherein said expression is (i) mediated by promoters derived from stress-inducible genes, and (ii) in a living transgenic mouse, said method comprising

(a) administering the analyte to a living transgenic mouse of claim 38, wherein administering of said analyte is carried out under conditions that permit light generation mediated by said light generating polypeptide,

(b) placing the transgenic mouse within a detection field of a photo detector device,

(c) maintaining the transgenic mouse in the detection field of the device, and

(d) during said maintaining, measuring photon emission from the transgenic mouse with the photo detector device to detect the level of expression of the light generating polypeptide in the living transgenic mouse wherein said expression is mediated by at least one of the promoters.

46. (previously presented): The method of claim 45, further comprising,

(e) repeating steps (b) through (d) at selected intervals, wherein said repeating is effective to detect changes in the level of the light emission in the transgenic mouse over time.

47-48. (canceled)

49. (previously presented): A method of providing a transgenic mouse suitable for screening a selected analyte, comprising

generating a transgenic mouse of claim 38, and

providing said transgenic mouse or progeny thereof for use in screening a selected analyte.

50-64. (canceled)

65. (previously presented): The transgenic mouse of claim 38, wherein the method further comprises

introducing a third expression cassette comprising a promoter derived from a third stress-inducible gene into a mouse at an embryonic stage, said third promoter operably linked to sequences encoding a third light generating polypeptide and said third promoter derived from a different stress-inducible gene than said first and second promoters.

66. (previously presented): The transgenic mouse of claim 65, wherein (i) said first, second, and third promoters are each derived from a different gene, and (ii) said first, second, and third light generating polypeptides produce the same color of light.

67. (previously presented): The transgenic mouse of claim 65, wherein (i) said first, second, and third promoters are each derived from a different gene, and (ii) at least two of said first, second, and third light generating polypeptides produce different colors of light.

68. (previously presented): The transgenic mouse of claim 65, said panel further comprising additional expression cassettes, wherein each expression cassette comprises a promoter derived from a different stress-inducible gene, said promoter operably linked to sequences encoding a light generating polypeptide.

69-80. (canceled).

EVIDENCE APPENDIX

The following documents are submitted in the Evidence Appendix:

(1) U.S. Patent No. 5,650,135, which was submitted in an IDS dated August 21, 2001 (reference AA-1) and cited by the Office on a PTO-form 892 attached to an Office Action mailed on February 1, 2001;

(2) U.S. Patent No. 6,217,847, which was submitted in an IDS dated August 21, 2001 (reference AA-3) and considered by the Office on September 4, 2001;

(3) Declaration of David P. West Pursuant to 37 C.F.R. § 1.132, submitted with a response mailed on June 11, 2002 and considered in the Office Action mailed on August 27, 2002; and

(4) Abstract of Jankowsky et al. (2001) *Biomol. Eng* 17(6):157-165, submitted as Appendix to Declaration of David P. West on June 11, 2002 and considered in the Office Action mailed on August 27, 2002.

US005650135A

United States Patent [19]

Contag et al.

[11] Patent Number: 5,650,135

[45] Date of Patent: Jul. 22, 1997

[54] **NON-INVASIVE LOCALIZATION OF A LIGHT-EMITTING CONJUGATE IN A MAMMAL**

[75] Inventors: **Christopher H. Contag**; **Pamela R. Contag**, both of Mountain View; **David A. Benaron**, Redwood City, all of Calif.

[73] Assignee: **The Board of Trustees of the Leland Stanford Junior University**, Stanford, Calif.

[21] Appl. No.: **270,631**

[22] Filed: **Jul. 1, 1994**

[51] **Int. Cl.**[6] **A01K 49/00**; A01K 39/385; G01N 33/569; C07H 21/02

[52] **U.S. Cl.** **424/9.1**; 424/9.61; 424/193.1; 424/258.1; 424/93.2; 435/7.35; 435/7.9; 435/8; 435/172.3; 436/800; 536/25.32

[58] **Field of Search** 424/9, 9.1, 193.1, 424/258.1, 93.2, 9.6, 9.61; 436/800; 536/25.32; 435/7.35, 7.9, 968, 172.3, 8

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Primary Examiner—James C. Housel
Assistant Examiner—Jennifer Shaver
Attorney, Agent, or Firm—Pennie & Edmonds

[57] **ABSTRACT**

Methods and compositions for detecting and localizing light originating from a mammal are disclosed. Also disclosed are methods for targeting light emission to selected regions, as well as for tracking entities within the mammal. In addition, animal models for disease states are disclosed, as are methods for localizing and tracking the progression of disease or a pathogen within the animal, and for screening putative therapeutic compounds effective to inhibit the disease or pathogen.

20 Claims, 12 Drawing Sheets
(8 of 12 Drawing(s) in Color)

FIG. 1A

lux genes

aldehyde biosynthesis

aldehyde biosynthesis luciferase biosynthesis

B/Sa E HM H BsCXHC E C S B/Sa

FIG. 1B

SacI *EcoRI*

GAGCTCGAATTC

SmaI

XbaI *BamHI* *KpnI*

TCGACTCTAGAGGATCCCCGGGTACCG

SalI *PstI*

HindIII *SphI*

CCAAGCTTGCATGCCTGCAGGT

MCS

To Fig. 1c

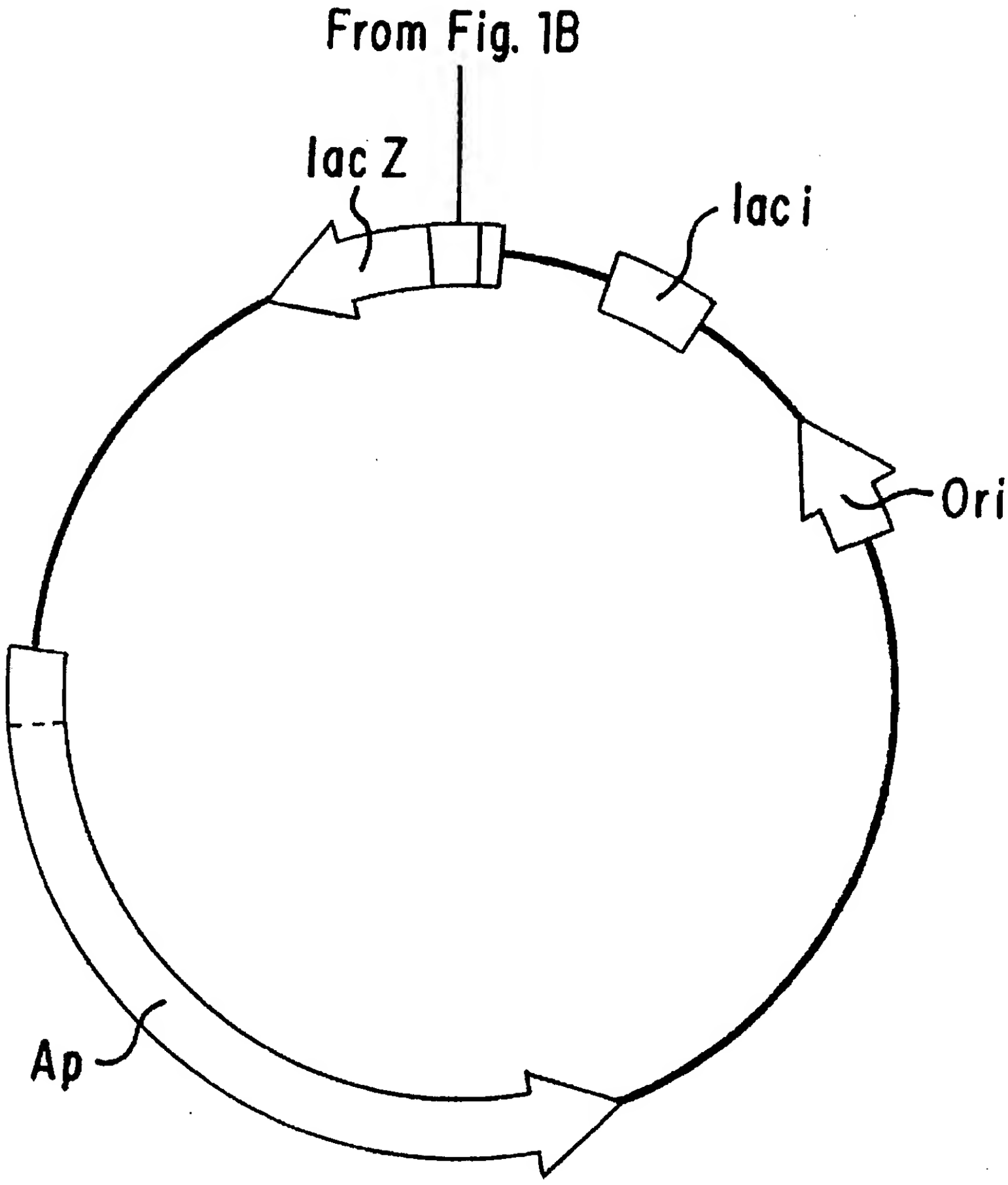


FIG. 1C

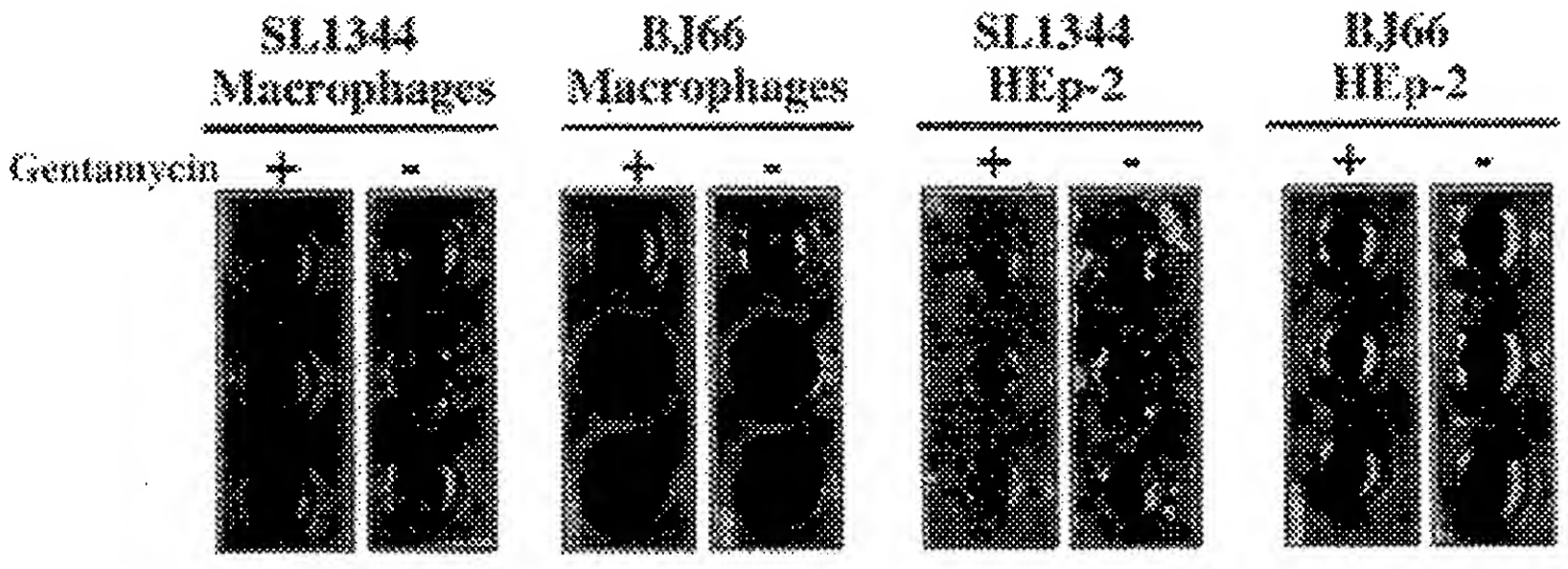

SL1344
Macrophages
BJ66
Macrophages
SL1344
HEp-2
BJ66
HEp-2
Gentamycin
+
-
+
-
+
-
+
-

Fig. 2A

FIG. 2D

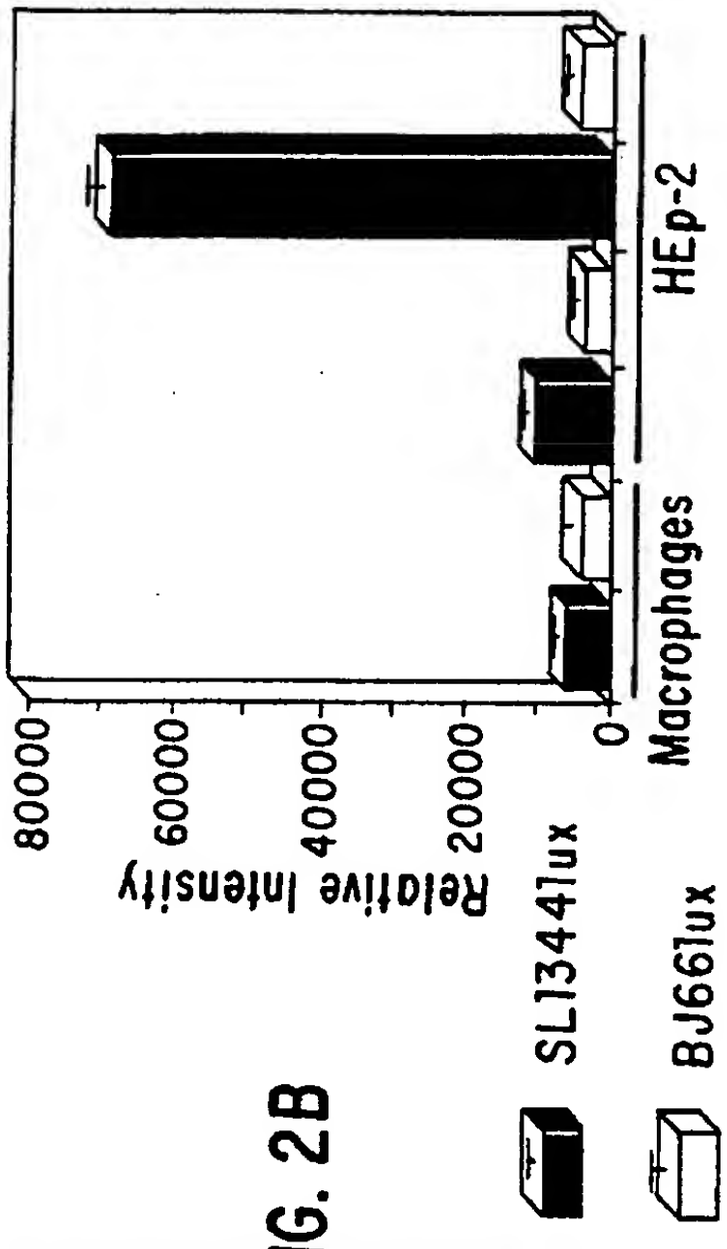


FIG. 2E

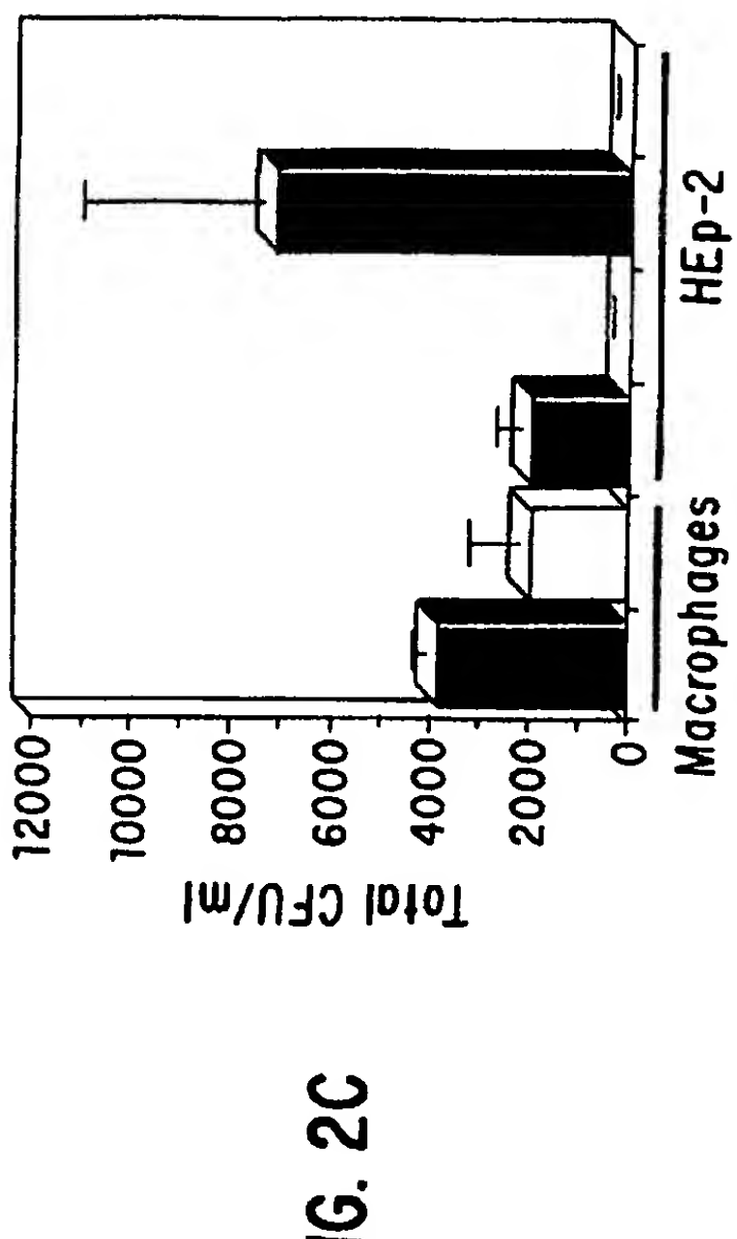


FIG. 2B

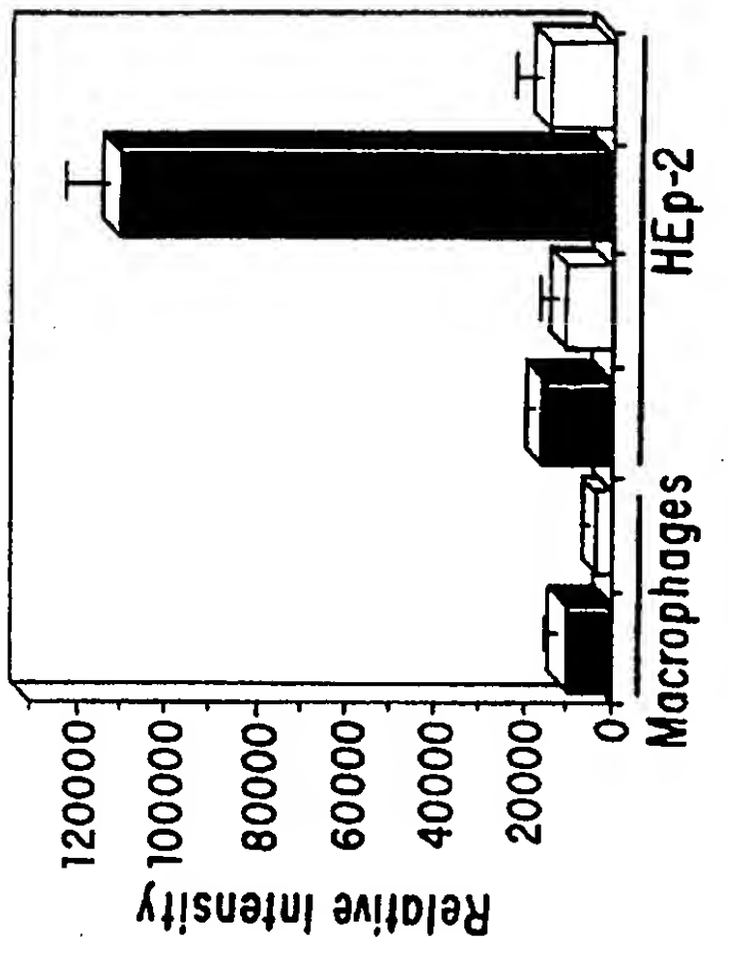
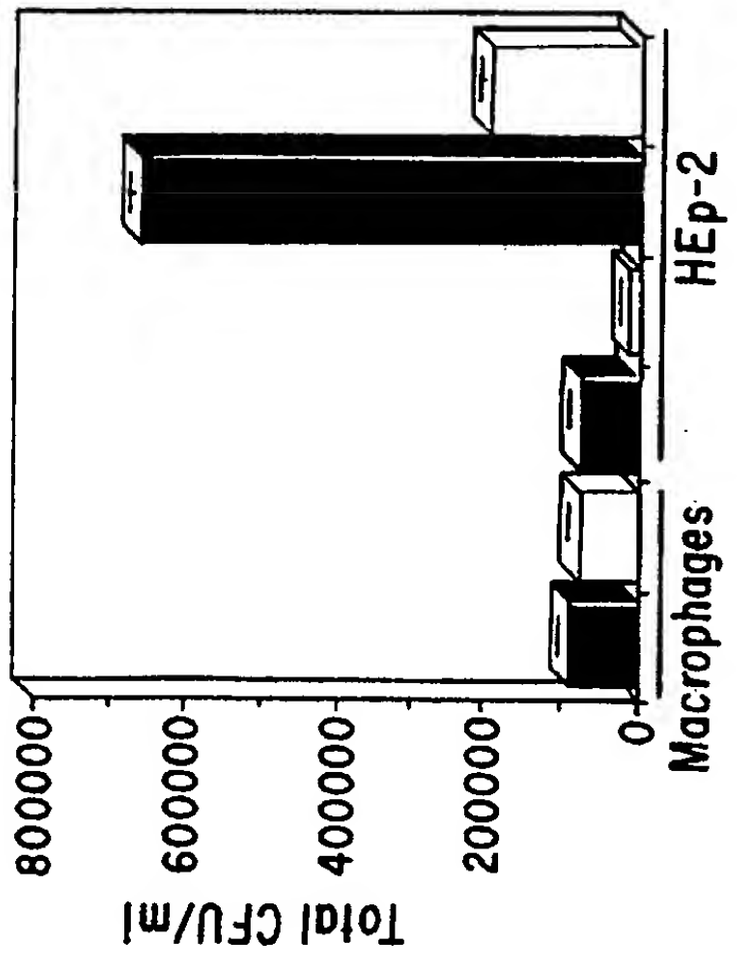


FIG. 2C



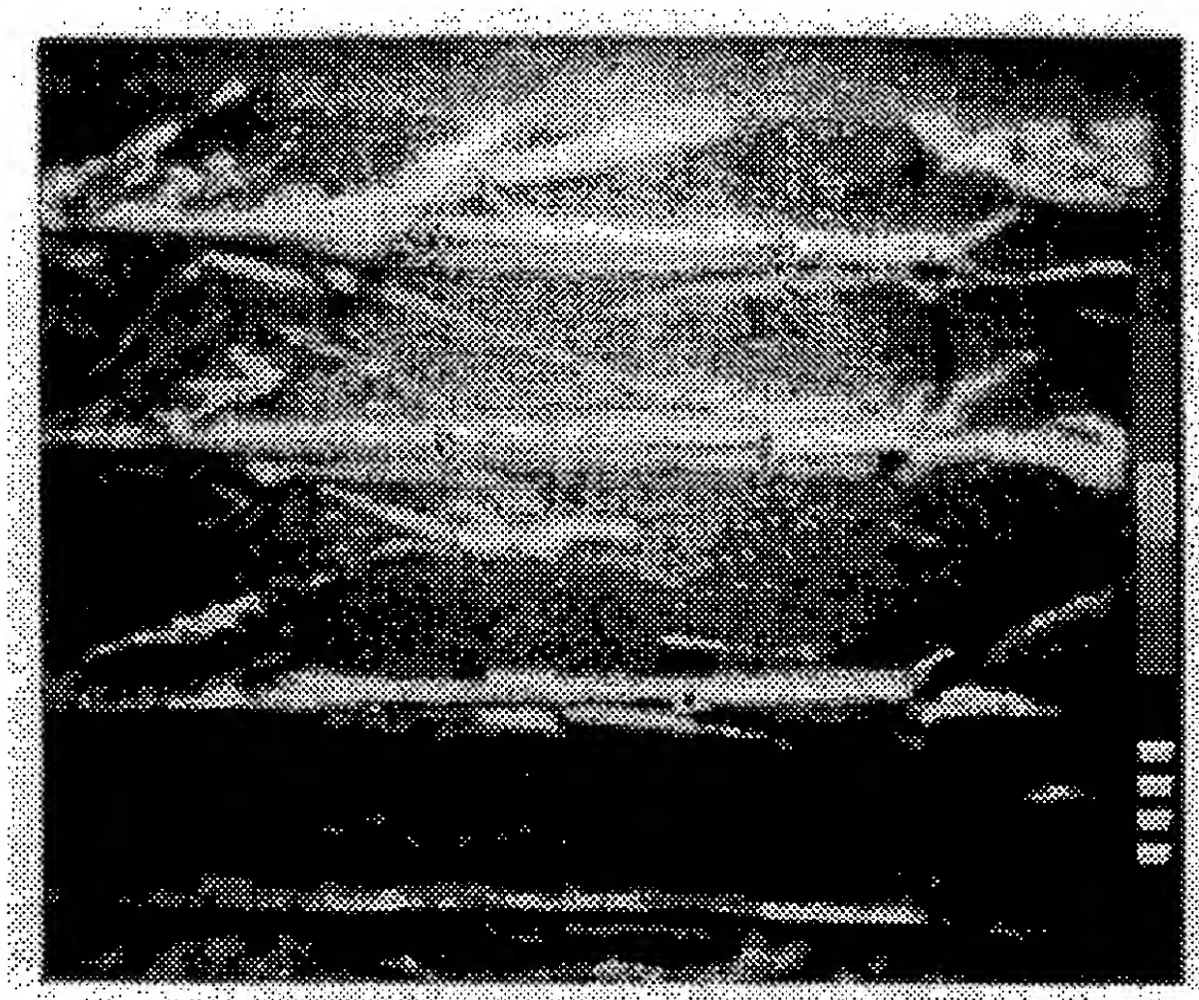


Fig. 3

Fig. 6

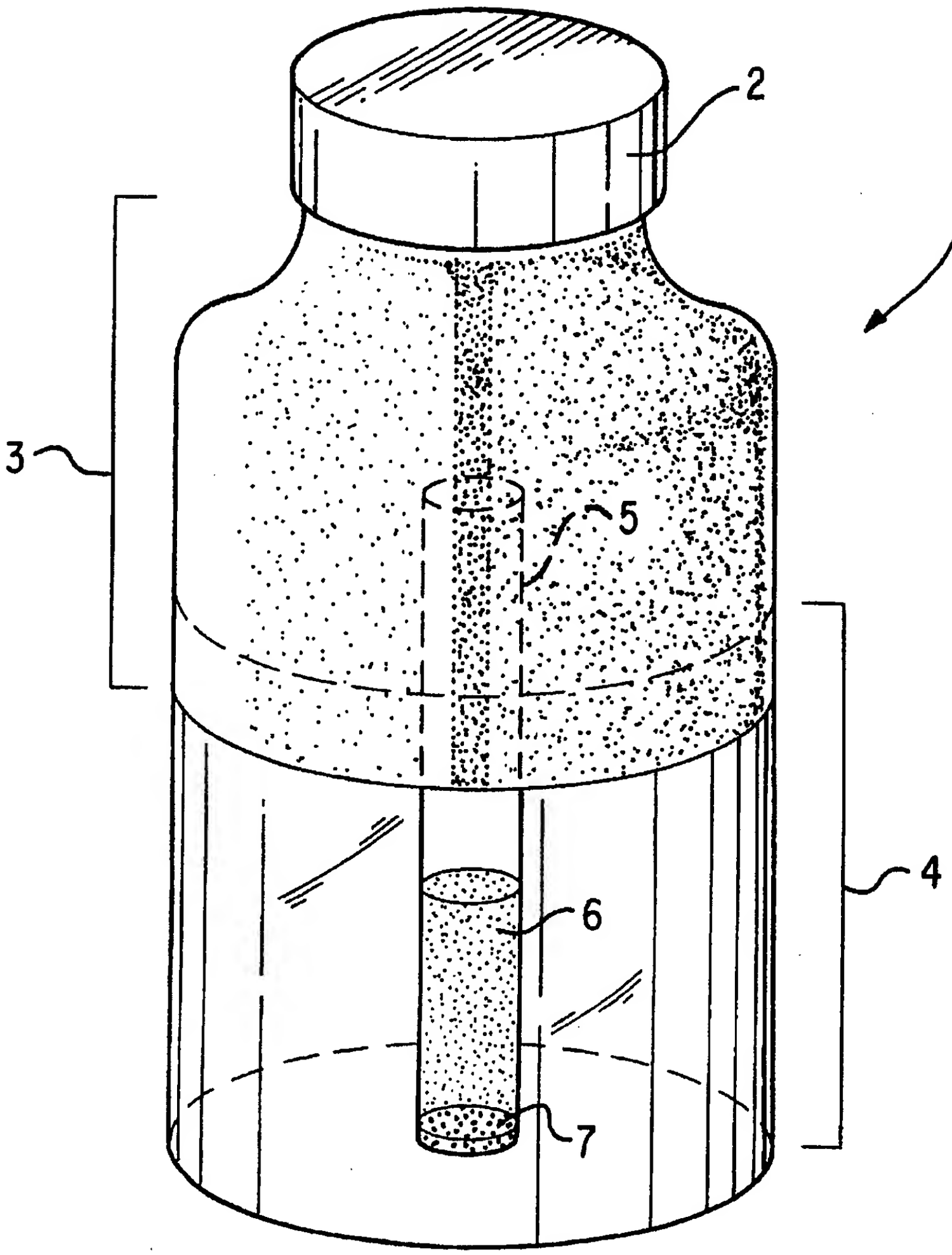


FIG. 4

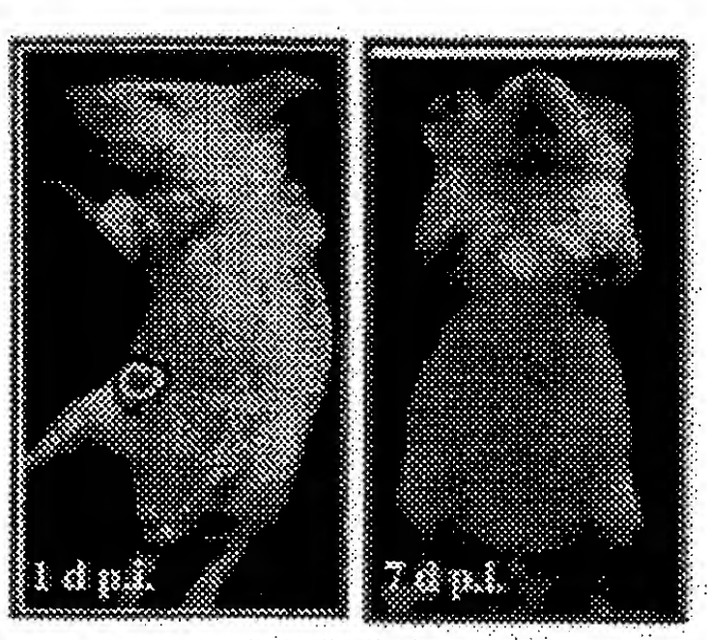
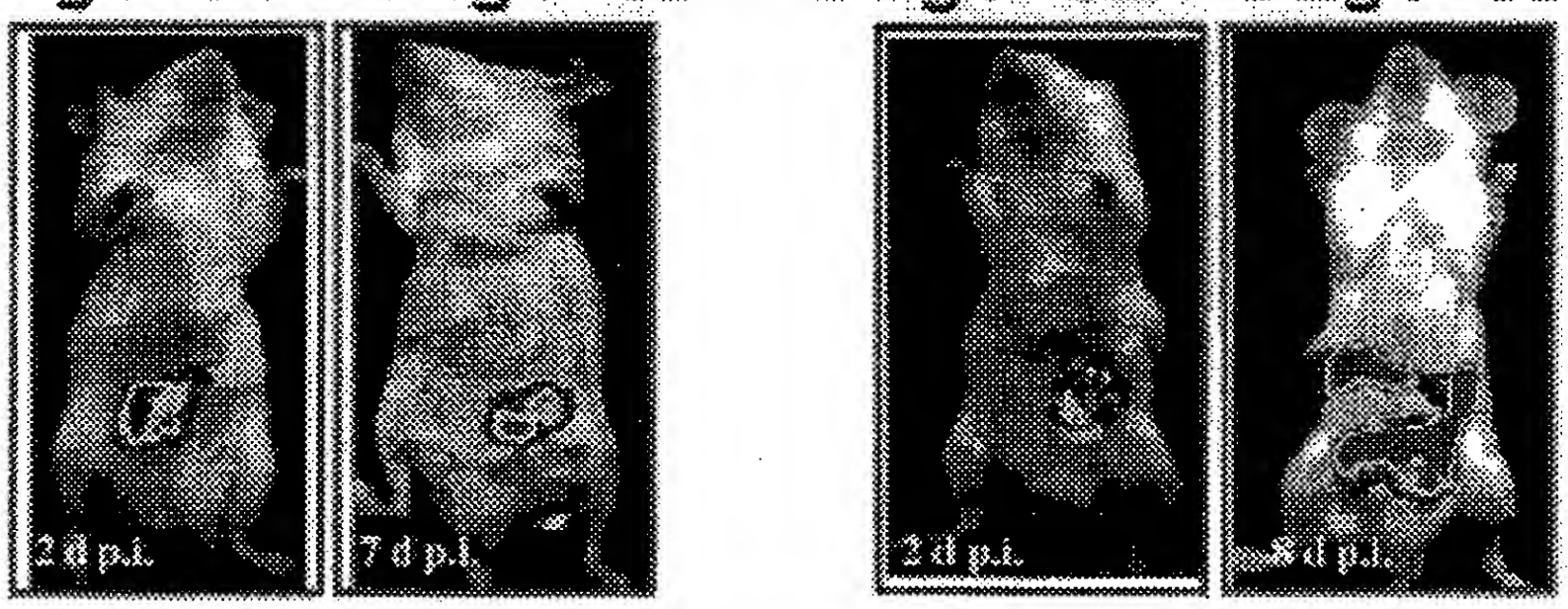


Fig. 5A Fig. 5B

LR5000lux

Fig. 5C Fig. 5D Fig. 5E Fig. 5F

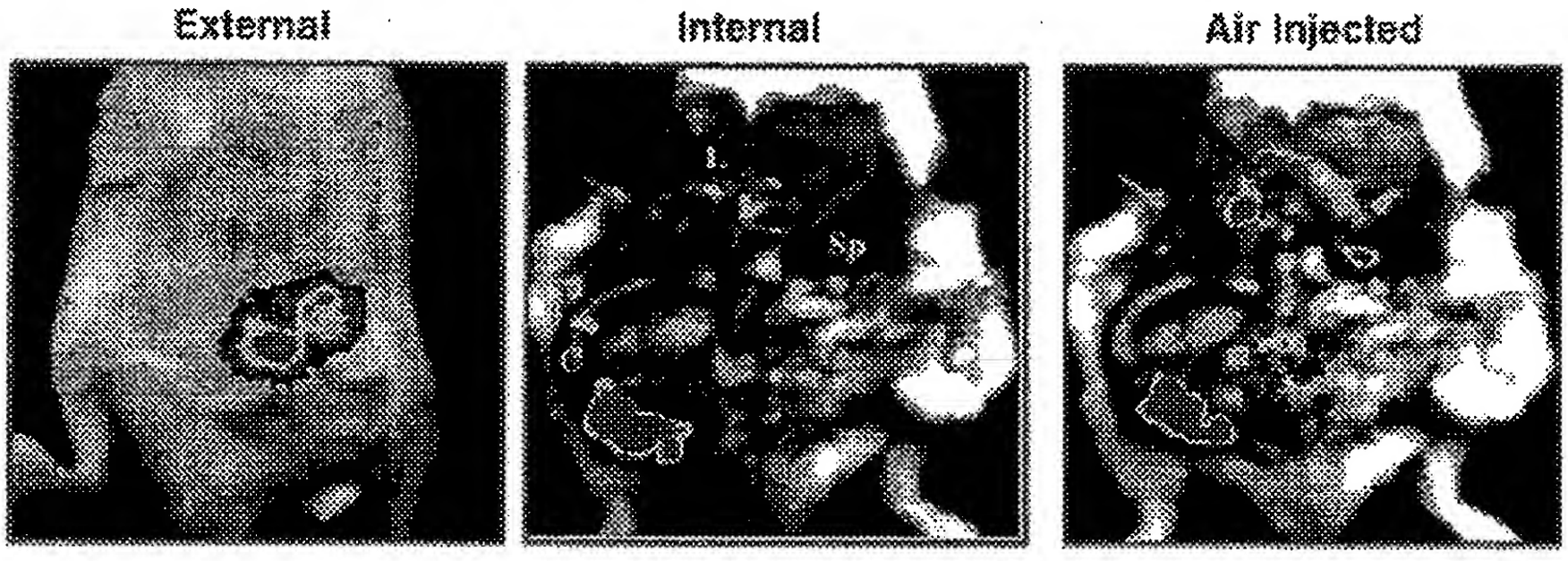


RJ66lux SL1344lux

Fig. 7A

Fig. 7B

Fig. 8A Fig. 8B Fig. 8C



BJ66lux Infection (7 d p.i.)

Fig. 9A

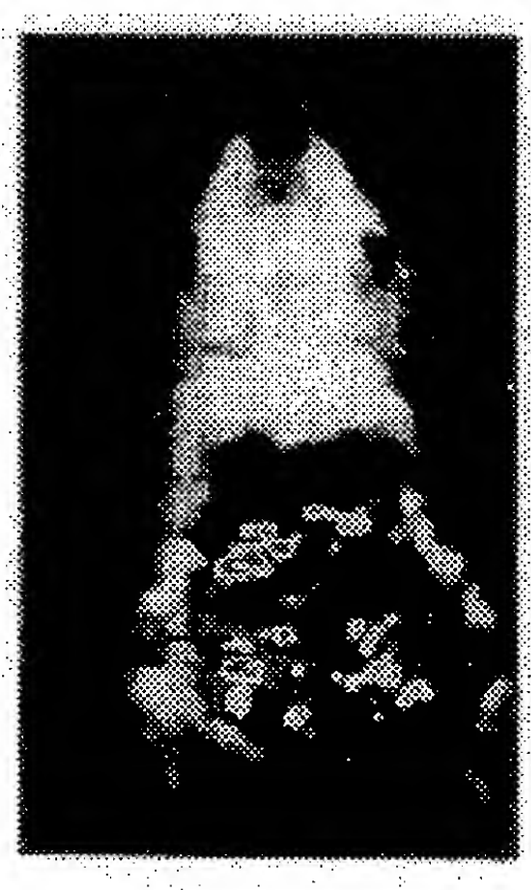


Fig. 9B

Fig. 9C

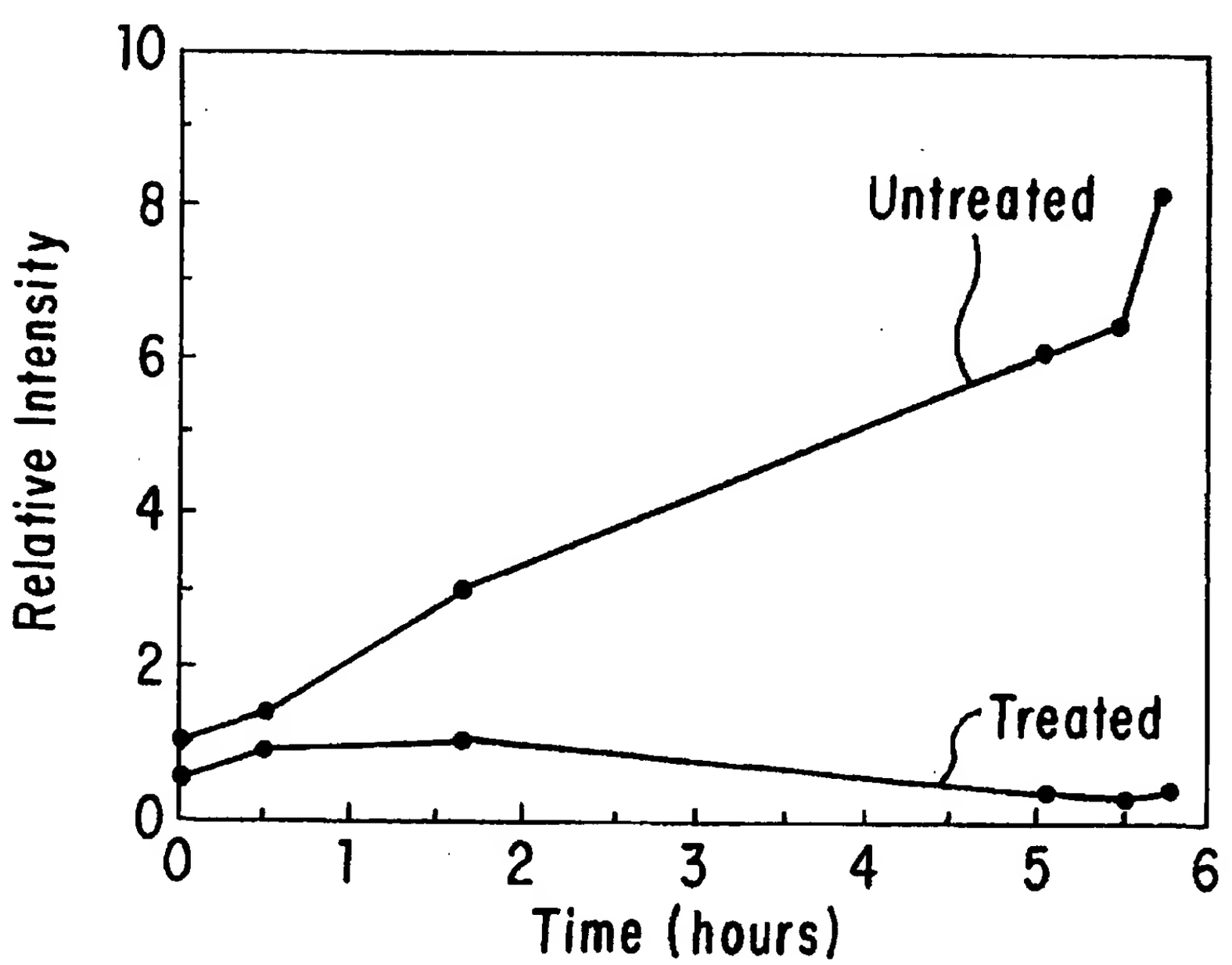

Relative Intensity
Time (hours)
Untreated
Treated
0
2
4
6
8
10
0
1
2
3
4
5
6

FIG. 10A

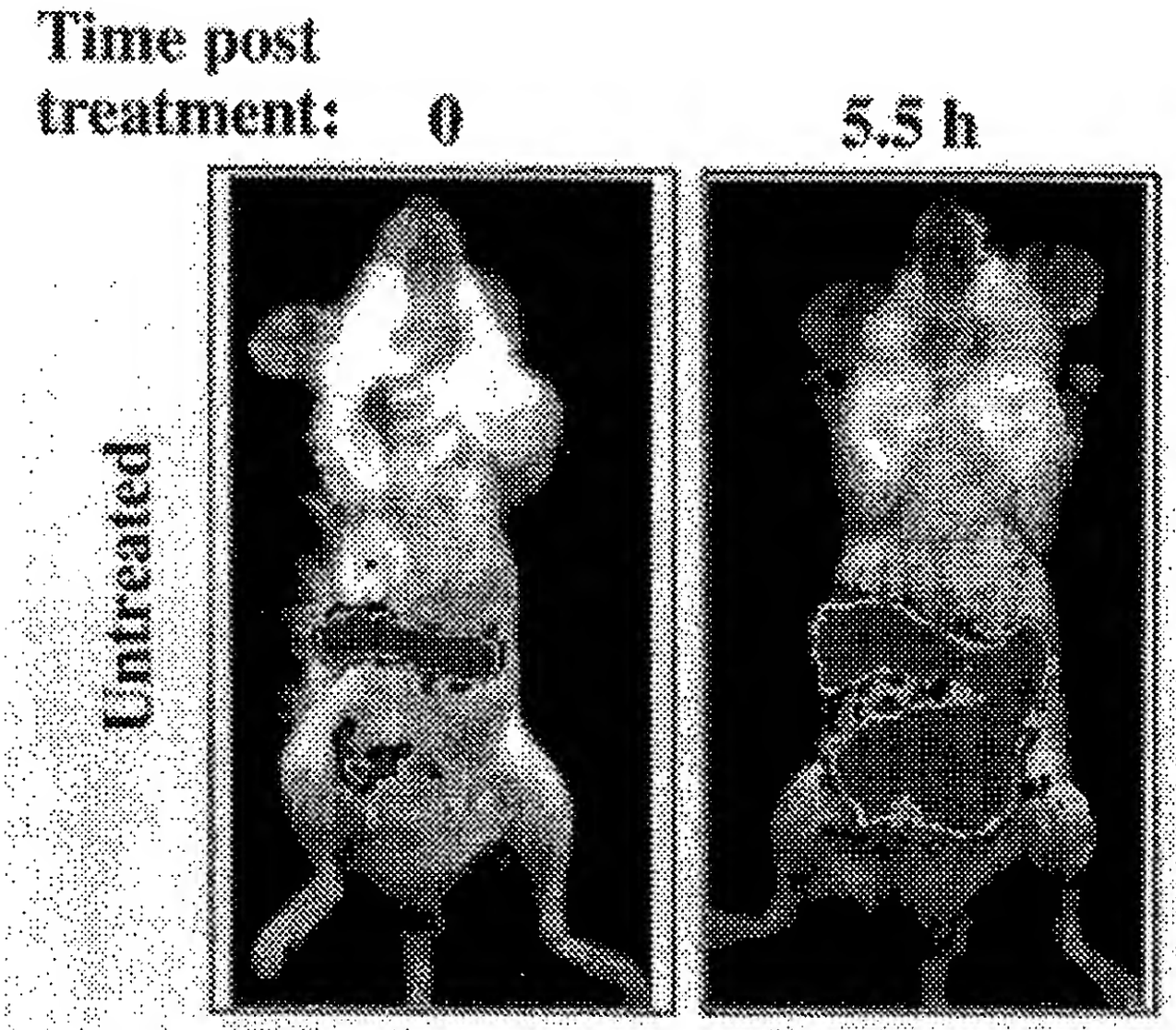


Fig. 10B Fig. 10C

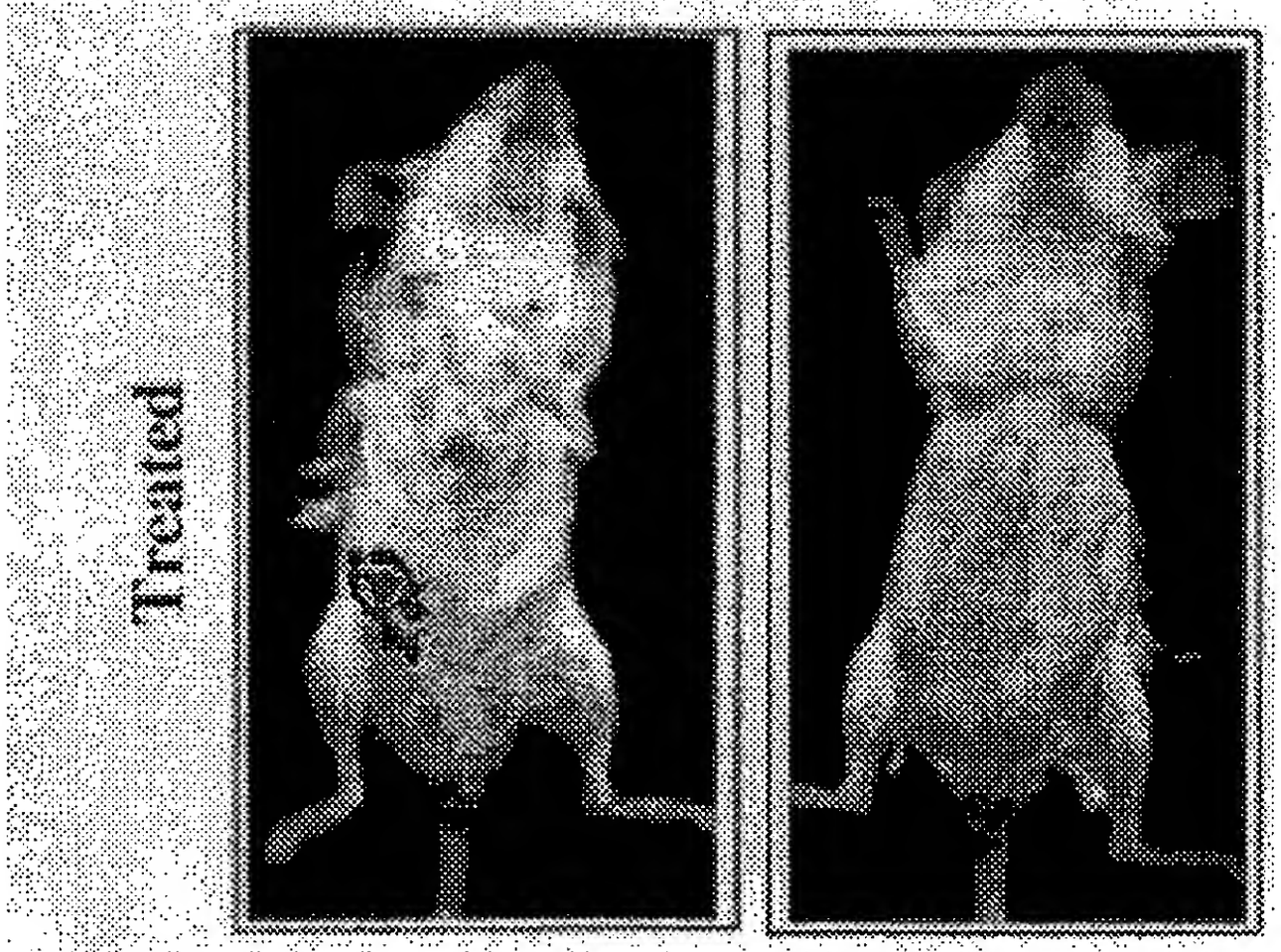


Fig. 10D Fig. 10E

NON-INVASIVE LOCALIZATION OF A LIGHT-EMITTING CONJUGATE IN A MAMMAL

FIELD OF THE INVENTION

The present invention relates to non-invasive methods and compositions for detecting, localizing and tracking light-emitting entities and biological events in a mammalian subject.

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BACKGROUND OF THE INVENTION

The ability to monitor the progression of infectious diseases is limited by the current ex vivo methods of detecting and quantifying infectious agents in tissues. The replication of an infectious agent in a host often involves primary, secondary and tertiary sites of replication. The sites of replication and the course that an infectious agent follows through these sites is determined by the route of inoculation, factors encoded by the host as well as determinants of the infecting agent.

Experience may offer, in some cases, an estimate of probable sites of replication and the progress of an infection. It is more often the case, however, that the sites of infection, and the pace of the disease are either not known or can only roughly be estimated. Moreover, the progression of an infectious disease, even in inbred strains of mice, is often individualized, and serial, ex vivo analyses of many infected animals need to be conducted to determine, on the average, what course a disease will follow in an experimentally infected host.

Accordingly, it would be desirable to have a means of tracking the progression of infection in an animal model. Ideally, the tracking could be done non-invasively, such that a single animal could be evaluated as often as necessary without detrimental effects. Methods and compositions of the present invention provide a non-invasive approach to detect, localize and track a pathogen, as well as other entities, in a living host, such as a mammal.

SUMMARY OF THE INVENTION

In one embodiment, the invention includes a noninvasive method for detecting the localization of a biocompatible entity in a mammalian subject. The entity can be a molecule, macromolecule, cell, microorganism (including a pathogen), a particle, or the like.

The method includes administering to the subject a conjugate of the entity and a light-generating moiety. Light-generating moieties are typically molecules or macromolecules that give off light. They may generate light as a result of radiation absorption (e.g. fluorescent or phosphorescent

molecules), or as a result of a chemical reaction (e.g. bioluminescent proteins). Exemplary light-generating moieties are bioluminescent proteins, such as luciferase and aequorin, and colored or fluorescent proteins, such as yellow fluorescent protein and ferredoxin IV.

The moiety may be conjugated to the entity by a variety of techniques, including incorporation during synthesis of the entity (e.g. chemical or genetic, such a fusion protein of an antibody fragment and a light-generating protein), chemical coupling post-synthesis, non-covalent association (e.g. encapsulation by liposomes), in-situ synthesis in the entity (e.g. expression of a heterologous bioluminescent protein in a transformed cell), or in situ activatable promoter-controlled expression of a bioluminescent protein in cells of a transgenic animal stimulated by a promoter inducer (e.g. interferon-activated promoter stimulated by infection with a virus).

After a period of time in which the conjugate can localize in the subject, the subject is immobilized within the detection field of a photodetector device for a period of time effective to measure a sufficient amount of photon emission (with the photodetector device) to construct an image. An exemplary photodetector device is an intensified charge-coupled device (ICCD) camera coupled to an image processor. If the image can be constructed in a time short relative to the time scale at which an "unimmobilized" subject moves, the subject is inherently "immobilized" during imaging and no special immobilization precautions are required. An image from the photon emission data is then constructed.

The method described above can be used to track the localization of the entity in the subject over time, by repeating the imaging steps at selected intervals and constructing images corresponding to each of those intervals.

The method described above can be used in a number of specific applications, by attaching, conjugating or incorporating targeting moieties onto the entity. The targeting moiety may be an inherent property of the entity (e.g. antibody or antibody fragment), or it may be conjugated to, attached to, or incorporated in the entity (e.g. liposomes containing antibodies). Examples of targeting moieties include antibodies, antibody fragments, enzyme inhibitors, receptor-binding molecules, various toxins and the like. Targets of the targeting moiety may include sites of inflammation, infection, thrombotic plaques and tumor cells. Markers distinguishing these targets, suitable for recognition by targeting moieties, are well known.

Further, the method may be used to detect and localize sites of infection by a pathogen in an animal model, using the pathogen (e.g. Salmonella) conjugated to a light-generating moiety as the entity.

In a related embodiment, the invention includes a noninvasive method for detecting the level of a biocompatible entity in a mammalian subject over time. The method is similar to methods described above, but is designed to detect changes in the level of the entity in the subject over time, without necessarily localizing the entity in the form of an image. This method is particularly useful for monitoring the effects of a therapeutic substance, such an antibiotic, on the levels of an entity, such as a light-emitting bacterium, over time.

In another embodiment, the invention includes a noninvasive method for detecting the integration of a transgene in a mammalian subject. The method includes administering to the subject, a vector construct effective to integrate a transgene into mammalian cells. Such constructs are well known in the art. In addition to the elements necessary to integrate effectively, the construct contains a transgene (e.g. a therapeutic gene), and a gene encoding a light-generating protein under the control of a selected activatable promoter. After a period of time in which the construct can achieve integration, the promoter is activated. For example, if an interferon promoter is used, a poly-inosine and -cytosine duplex (poly-IC) can be locally administered (e.g. footpad injection) to stimulate interferon production. The subject is then placed within the detection field of a photodetector device, such as an individual wearing light-intensifying "night vision" goggles, and the level of photon emission is measured, or evaluated. If the level is above background (i.e. if light can be preferentially detected in the "activated" region), the subject is scored as having integrated the transgene.

In a related embodiment, the invention includes a noninvasive method for detecting the localization of a promoter-induction event in an animal made transgenic or chimeric for a construct including a gene encoding a light-generating protein under the control of an inducible promoter. Promoter induction events include the administration of a substance which directly activates the promoter, the administration of a substance which stimulates production of an endogenous promoter activator (e.g. stimulation of interferon production by RNA virus infection), the imposition of conditions resulting in the production of an endogenous promoter activator (e.g. heat shock or stress), and the like. The event is triggered, and the animal is imaged as described above.

In yet another embodiment, the invention includes pathogens, such as Salmonella, transformed with a gene expressing a light-generating protein, such as luciferase.

In another aspect, the invention includes a method of identifying therapeutic compounds effective to inhibit spread of infection by a pathogen. The method includes administering a conjugate of the pathogen and a light-generating moiety to control and experimental animals, treating the experimental animals with a putative therapeutic compound, localizing the light-emitting pathogen in both control and experimental animals by the methods described above, and identifying the compound as therapeutic if the compound is effective to significantly inhibit the spread or replication of the patbogen in the experimental animals relative to control animals. The conjugates include a fluorescently-labeled antibodies, fluorescently-labeled particles, fluorescently-labeled small molecules, and the like.

In still another aspect, the invention includes a method of localizing entities conjugated to light-generating moieties through media of varying opacity. The method includes the use of photodetector device to detect photons transmitted through the medium, integrate the photons over time, and generate an image based on the integrated signal.

In yet another embodiment, the invention includes a method of measuring the concentration of selected substances, such as dissolved oxygen or calcium, at specific sites in an organism. The method includes entities, such as cells, containing a concentration sensor—a light-generating molecule whose ability to generate light is dependent on the concentration of the selected substance. The entity containing the light-generating molecule is administered such that it adopts a substantially uniform distribution in the animal or in a specific tissue or organ system (e.g. spleen). The organism is imaged, and the intensity and localization of light emission is correlated to the concentration and location of the selected substance. Alternatively, the entity contains a

second marker, such as a molecule capable of generating light at a wavelength other than the concentration sensor. The second marker is used to normalize for any non-uniformities in the distribution of the entity in the host, and thus permit a more accurate determination of the concentration of the selected substance.

In another aspect, the invention includes a method of identifying therapeutic compounds effective to inhibit the growth and/or the metastatic spread of a tumor. The method includes (i) administering tumor cells labeled with or containing light-generating moieties to groups of experimental and control animals, (ii) treating the experimental group with a selected compound, (iii) localizing the tumor cells in animals from both groups by imaging photon emission from the light-generating molecules associated with the tumor cells with a photodetector device, and (iv) identifying a compound as therapeutic if the compound is able to significantly inhibit the growth and/or metastatic spread of the tumor in the experimental group relative to the control group.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawings will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

FIGS. **1**A, 1B and 1C show a map of the lux pCGLS1 plasmid used to transform salmonella strains SL1344, BJ66 and LB5000 to generate strains SL1344lux, BJ66lux and LB5000lux.

FIGS. **2**A–E show the results of assays to measure adherence and invasion, by Salmonella strains SL1344lux and BJ66lux, of macrophages and HEp-2 cells. FIG. 2A shows luminescent bacterial cells localized in wells of an assay dish. The pseudo-color image, obtained by integrating photons over one minute, is superimposed over a gray scale image of the assay dish, producing the "composite image" shown. FIG. 2B shows the relative light intensity of wells that were not treated with gentamicin. FIG. 2C shows the number of colony forming units (CFU) per ml isolated from the same wells as were imaged in FIG. 2B. FIG. 2D shows the relative light intensity of wells that were treated with gentamicin. FIG. 2E shows the number of colony forming units (CFU) per ml isolated from the same wells as were imaged in FIG. 2D.

FIG. **3** is a composite image of four glass capillary tubes containing dilutions of LB5000lux bacterial suspensions. Luminescence was determined by integrating over 30 seconds. Air pockets are present in each tube on both sides of the suspension.

FIG. **4** is a schematic diagram of a vial used to test the transmission of light generated by LB5000lux through animal tissue.

FIGS. **5**A–F show composite images of Balb/c mice orally inoculated with low virulence LB5000lux (FIGS. 5A–B), non-invasive BJ66lux (FIGS. 5C–D) and virulent SL1344lux (FIGS. 5E–F) salmonella, and imaged at the times indicated in the figure. The luminescence signal was integrated over 5 minutes.

FIG. **6** is a composite image showing the distribution of salmonella in mice 32 hours following intraperitoneal (i.p.) injections with either virulent SL1344lux (left two animals) or low virulence LB5000lux (right two animals) strains of the bacterium.

FIGS. 7A and 7B show the distribution of virulent salmonella in mice resistant to systemic salmonella infections (129×Balb/c, Ity$^{r/s}$). FIG. 7A—day 1, FIG. 7B—day 8.

FIGS. 8A–C show the distribution of mutant salmonella with reduced virulence (BJ66lux) seven days following oral inoculation. FIG. 8A shows external, non-invasive imaging of the luminescence. FIG. 8B shows the same animal imaged following laparotomy. Labeled organs are C—cecum, L—liver, I—small intestine and Sp—spleen. FIG. 8C shows a post-laparotomy image generated following injection of air into the lumen of the intestine both anterior and posterior to the cecum.

FIGS. 9A, 9B and 9C show the distribution of salmonella SL1344lux in susceptible Balb/c mice following intraperitoneal inoculation with SL1344lux. FIG. 9A was imaged prior to the opening of the peritoneal cavity. FIG. 9B was imaged after the opening of the peritoneal cavity, and FIG. 9C was imaged after the cecum was pulled to the left side.

FIGS. 10A–E show the effects of ciprofloxacin treatment on bioluminescence from SL1344lux salmonella in orally-inoculated mice. FIG. 10A shows a graph of the relative bioluminescence intensity, measured from the abdominal area, as a function of time after initiation of treatment, for treated and untreated animals. FIGS. 10B and 10D show composite images of mice 8 days after oral inoculation with SL1344lux salmonella, before treatment with ciprofloxacin. FIGS. 10C and 10E show composite images of the same mice 5.5 hours either following treatment (FIG. 10E) or control (no treatment; FIG. 10C).

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art of the present invention.

Opaque medium is used herein to refer to a medium that is "traditionally" opaque, not necessarily absolutely opaque. Accordingly, an opaque medium is defined as a medium that is commonly considered to be neither transparent nor translucent, and includes items such as a wood board, and flesh and skin of a mammal.

Luciferase, unless stated otherwise, includes prokaryotic and eukaryotic luciferases, as well as variants possessing varied or altered optical properties, such as luciferases that luminesce at wavelengths in the red range.

Biocompatible entity is an entity that can be administered to a mammal. This includes pathogens which may be deleterious to the mammal. In reference to an animal whose cells contain a transgene expressing a light-generating protein, biocompatible entity refers to the transgene-containing cells comprising the mammal.

Light-generating is defined as capable of generating light through a chemical reaction or through the absorption of radiation.

Light is defined herein, unless stated otherwise, as electromagnetic radiation having a wavelength of between about 300 nm and about 1100 nm.

Spread of infection typically refers to the spreading and colonization by a pathogen of host sites other than the initial infection site. The term can also include, however, growth in size and/or number of the pathogen at the initial infection site.

lux—prokaryotic genes associated with luciferase and photon emission.

luc—eukaryotic genes associated with luciferase and photon emission.

Promoter induction event refers to an event that results in the direct or indirect induction of a selected inducible promoter.

Heterologous gene refers to a gene which has been transfected into a host organism. Typically, a heterologous gene refers to a gene that is not originally derived from the transfected or transformed cells' genomic DNA.

II. General Overview of the Invention

The present invention includes methods and compositions relating to non-invasive imaging and/or detecting of light-emitting conjugates in mammalian subjects. The conjugates contain a biocompatible entity and a light-generating moiety. Biocompatible entities include, but are not limited to, small molecules such as cyclic organic molecules; macromolecules such as proteins; microorganisms such as viruses, bacteria, yeast and fungi; eukaryotic cells; all types of pathogens and pathogenic substances; and particles such as beads and liposomes. In another aspect, biocompatible entities may be all or some of the cells that constitute the mammalian subject being imaged.

Light-emitting capability is conferred on the entities by the conjugation of a light-generating moiety. Such moieties include fluorescent molecules, fluorescent proteins, enzymatic reactions giving off photons and luminescent substances, such as bioluminescent proteins. The conjugation may involve a chemical coupling step, genetic engineering of a fusion protein, or the transformation of a cell, microorganism or animal to express a bioluminescent protein. For example, in the case where the entities are the cells constituting the mammalian subject being imaged, the light-generating moiety may be a bioluminescent or fluorescent protein "conjugated" to the cells through localized, promoter-controlled expression from a vector construct introduced into the cells by having made a transgenic or chimeric animal.

Light-emitting conjugates are typically administered to a subject by any of a variety of methods, allowed to localize within the subject, and imaged. Since the imaging, or measuring photon emission from the subject, may last up to tens of minutes, the subject is usually, but not always, immobilized during the imaging process.

Imaging of the light-emitting entities involves the use of a photodetector capable of detecting extremely low levels of light—typically single photon events—and integrating photon emission until an image can be constructed. Examples of such sensitive photodetectors include devices that intensify the single photon events before the events are detected by a camera, and cameras (cooled, for example, with liquid nitrogen) that are capable of detecting single photons over the background noise inherent in a detection system.

Once a photon emission image is generated, it is typically superimposed on a "normal" reflected light image of the subject to provide a frame of reference for the source of the emitted photons (i.e. localize the light-emitting conjugates with respect to the subject). Such a "composite" image is then analyzed to determine the location and/or amount of a target in the subject.

The steps and embodiments outlined above are presented in greater detail, below.

III. Light-Emitting Entities

A. Light-Generating Moieties

The light-generating moieties (LGMs), molecules or constructs useful in the practice of the present invention may take any of a variety of forms, depending on the application. They share the characteristic that they are luminescent, that is, that they emit electromagnetic radiation in ultraviolet (UV), visible and/or infra-red (IR) from atoms or molecules as a result of the transition of an electronically excited state to a lower energy state, usually the ground state.

Examples of light-generating moieties include photoluminescent molecules, such as fluorescent molecules, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds.

Two characteristics of LGMs that bear considerable relevance to the present invention are their size and their spectral properties. Both are discussed in the context of specific types of light-generating moieties described below, following a general discussion of spectral properties.

1. Spectral Properties. An important aspect of the present invention is the selection of light-generating moieties that produce light capable of penetrating animal tissue such that it can be detected externally in a non-invasive manner. The ability of light to pass through a medium such as animal tissue (composed mostly of water) is determined primarily by the light's intensity and wavelength.

The more intense the light produced in a unit volume, the easier the light will be to detect. The intensity of light produced in a unit volume depends on the spectral characteristics of individual LGMs, discussed below, and on the concentration of those moieties in the unit volume. Accordingly, conjugation schemes that place a high concentration of LGMs in or on an entity (such as high-efficiency loading of a liposome or high-level expression of a bioluminescent protein in a cell) typically produce brighter light-emitting conjugates (LECs), which are easier to detect through deeper layers of tissue, than schemes which conjugate, for example, only a single LGM onto each entity.

A second factor governing the detectability of an LGM through a layer of tissue is the wavelength of the emitted light. Water may be used to approximate the absorption characteristics of animal tissue, since most tissues are composed primarily of water. It is well known that water transmits longer-wavelength light (in the red range) more readily than it does shorter wavelength light.

Accordingly, LGMs which emit light in the range of yellow to red (550–1100 nm) are typically preferable to LGMs which emit at shorter wavelengths. Several of the LGMs discussed below emit in this range. However, it will be noted, based on experiments performed in support of the present invention and presented below, that excellent results can be achieved in practicing the present invention with LGMs that emit in the range of 486 nm, despite the fact that this is not an optimal emission wavelength. These results are possible, in part, due to the relatively high concentration of LGMs (luciferase molecules) present in the LECs (transformed Salmonella cells) used in these experiments, and to the use of a sensitive detector. It will be understood that through the use of LGMs with a more optimal emission wavelength, similar detection results can be obtained with LGEs having lower concentrations of the LGMs.

2. Fluorescence-based Moieties. Fluorescence is the luminescence of a substance from a single electronically excited state, which is of very short duration after removal of the source of radiation. The wavelength of the emitted fluorescence light is longer than that of the exciting illumination (Stokes' Law), because part of the exciting light is converted into heat by the fluorescent molecule.

Because fluorescent molecules require input of light in order to luminesce, their use in the present invention may be more complicated than the use of bioluminescent molecules.

Precautions are typically taken to shield the excitatory light so as not to contaminate the fluorescence photon signal being detected from the subject. Obvious precautions include the placement of an excitation filter, such that employed in fluorescence microscope, at the radiation source. An appropriately-selected excitation filter blocks the majority of photons having a wavelength similar to that of the photons emitted by the fluorescent moiety. Similarly a barrier filter is employed at the detector to screen out most of the photons having wavelengths other than that of the fluorescence photons. Filters such as those described above can be obtained from a variety of commercial sources, including Omega Optical, Inc. (Brattleboro, Vt.).

Alternatively, a laser producing high intensity light near the appropriate excitation wavelength, but not near the fluorescence emission wavelength, can be used to excite the fluorescent moieties. An x-y translation mechanism may be employed so that the laser can scan the subject, for example, as in a confocal microscope.

As an additional precaution, the radiation source can be placed behind the subject and shielded, such that the only radiation photons reaching the site of the detector are those that pass all the way through the subject. Furthermore, detectors may be selected that have a reduced sensitivity to wavelengths of light used to excite the fluorescent moiety.

Through judicious application of the precautions above, the detection of fluorescent LGMs according to methods of the present invention is possible.

Fluorescent moieties include small fluorescent molecules, such as fluorescein, as well as fluorescent proteins, such as green fluorescent protein (Chalfie, et al., Morin, et al.) and lumazine and yellow fluorescent proteins (O'Kane, et al., Daubner, et al.). In addition, certain colored proteins such as ferredoxin IV (Grabau, et al.), whose fluorescence characteristics have not been evaluated, may be fluorescent and thus applicable for use with the present invention. Ferredoxin IV is a particularly promising candidate, as it has a reddish color, indicating that it may fluoresce or reflect at a relatively long wavelength and produce light that is effective at penetrating tissue. Furthermore, the molecule is small for a protein (95 amino acids), and can thus be conjugated to entities with a minimal impact on their function.

An advantage of small fluorescent molecules is that they are less likely to interfere with the bioactivity of the entity to which they are attached than a would a larger light-generating moiety. In addition, commercially-available fluorescent molecules can be obtained with a variety of excitation and emission spectra that are suitable for use with the present invention. For example, Molecular Probes (Eugene, Oreg.) sells a number of fluorophores, including Lucifer Yellow (abs. at 428 nm, and emits at 535 nm) and Nile Red (abs. at 551 nm and emits at 636 nm). Further, the molecules can be obtained derivatized with a variety of groups for use with various conjugation schemes (e.g. from Molecular Probes).

3. Bioluminescence-based Moieties. The subjects of chemiluminescence (luminescence as a result of a chemical reaction) and bioluminescence (visible luminescence from living organisms) have, in many aspects, been thoroughly studied (e.g., Campbell). A brief summary of salient features follows.

Bioluminescent molecules are distinguished from fluorescent molecules in that they do not require the input of radiative energy to emit light. Rather, bioluminescent molecules utilize chemical energy, such as ATP, to produce light. An advantage of bioluminescent moieties, as opposed to fluorescent moieties, is that there is virtually no background in the signal. The only light detected is light that is produced by the exogenous bioluminescent moiety. In contrast, the light used to excite a fluorescent molecule often results in the fluorescence of substances other than the intended target. This is particularly true when the "background" is as complex as the internal environment of a living animal.

Several types of bioluminescent molecules are known. They include the luciferase family (e.g. Wood, et al.) and the aequorin family (e.g. Prasher, et al.). Members of the luciferase family have been identified in a variety of prokaryotic and eukaryotic organisms. Luciferase and other enzymes involved in the prokaryotic luminescent (lux) systems, as well as the corresponding lux genes, have been isolated from marine bacteria in the Vibrio and Photobacterium genera and from terrestrial bacteria in the Xenorhabdus genus.

An exemplary eukaryotic organism containing a luciferase system (luc) is the North American firefly *Photinus pyralis*. Firefly luciferase has been extensively studied, and is widely used in ATP assays. cDNAs encoding luciferases from *Pyrophorus plagiophthalamus,* another species of click beetle, have been cloned and expressed (Wood, et al.). This beetle is unusual in that different members of the species emit bioluminescence of different colors. Four classes of clones, having 95–99% homology with each other, were isolated. They emit light at 546 nm (green), 560 nm (yellow-green), 578 nm (yellow) and 593 nm (orange). The last class (593 nm) may be particularly advantageous for use as a light-generating moiety with the present invention, because the emitted light has a wavelength that penetrates tissues more easily than shorter wavelength light.

Luciferases, as well as aequorin-like molecules, require a source of energy, such as ATP, NAD(P)H, and the like, and a substrate, such as luciferin or coelentrizine and oxygen.

The substrate luciferin must be supplied to the luciferase enzyme in order for it to luminesce. In those cases where a luciferase enzyme is introduced as an expression product of a vector containing cDNA encoding a lux luciferase, a convenient method for providing luciferin is to express not only the luciferase but also the biosynthetic enzymes for the synthesis of luciferin. In cells transformed with such a construct, oxygen is the only extrinsic requirement for bioluminescence. Such an approach, detailed in Example 1, is employed to generate lux-transformed Salmonella, which are used in experiments performed in support of the present invention and detailed herein.

The plasmid construct, encoding the lux operon obtained from the soil bacterium *Xenorhabdus luminescens* (Frackman, et al., 1990), confers on transformed *E coli* the ability to emit photons through the expression of the two subunits of the heterodimeric luciferase and three accessory proteins (Frackman, et al., 1990). Optimal bioluminescence for *E. Coli* expressing the lux genes of *X. luminescens* is observed at 37° C. (Szittner, et al., Xi, et al.) in contrast to the low temperature optima of luciferases from eukaryotic and other prokaryotic luminescent organisms (Campbell, 1988). The luciferase from *X. luminescens*, therefore, is well-suited for use as a marker for studies in animals.

Luciferase vector constructs such as the one described above and in Example 1, can be adapted for use in transforming a variety of host cells, including most bacteria, and many eukaryotic cells (luc constructs). In addition, certain viruses, such as herpes virus and vaccinia virus, can be genetically-engineered to express luciferase. For example, Kovacs, et al. teach the stable expression of the gene encoding firefly luciferase in a herpes virus. Brasier, et al.,

teach the use of luciferase gene constructs in mammalian cells. Luciferase expression from mammalian cells in culture has been studied using CCD imaging both macroscopically (Israel and Honigman, 1991) and microscopically (Hooper, et al., 1990).

B. Entities

The invention includes entities which have been modified or conjugated to include a light-generating moiety, construct or molecule, such as described above. Such conjugated or modified entities are referred to as light-emitting entities, light-emitting conjugates (LECs) or simply conjugates. The entities themselves may take the form of, for example, molecules, macromolecules, particles, microorganisms, or cells. The methods used to conjugate a light-generating moiety to an entity depend on the nature of the moiety and the entity. Exemplary conjugation methods are discussed in the context of the entities described below.

1. Small molecules. Small molecule entities which may be useful in the practice of the present invention include compounds which specifically interact with a pathogen or an endogenous ligand or receptor. Examples of such molecules include, but are not limited to, drugs or therapeutic compounds; toxins, such as those present in the venoms of poisonous organisms, including certain species of spiders, snakes, scorpions, dinoflagellates, marine snails and bacteria; growth factors, such as NGF, PDGF, TGF and TNF; cytokines; and bioactive peptides.

The small molecules are preferably conjugated to light-generating moieties that interfere only minimally, if at all, with the bioactivity of the small molecule, such as small fluorescent molecules (described above). Conjugations are typically chemical in nature, and can be performed by any of a variety of methods known to those skilled in the art.

The small molecule entity may be synthesized to contain a light-generating moiety, so that no formal conjugation procedure is necessary. Alternatively, the small molecule entity may be synthesized with a reactive group that can react with the light generating moiety, or vice versa.

Small molecules conjugated to light-generating moieties of the present invention may be used either in animal models of human conditions or diseases, or directly in human subjects to be treated. For example, a small molecule which binds with high affinity to receptor expressed on tumor cells may be used in an animal model to localize and obtain size estimates of tumors, and to monitor changes in tumor growth or metastasis following treatment with a putative therapeutic agent. Such molecules may also be used to monitor tumor characteristics, as described above, in cancer patients.

2. Macromolecules. Macromolecules, such as polymers and biopolymers, constitute another example of entities useful in practicing the present invention. Exemplary macromolecules include antibodies, antibody fragments, fusion proteins and certain vector constructs.

Antibodies or antibody fragments, purchased from commercial sources or made by methods known in the art (Harlow), can be used to localize their antigen in a mammalian subject by conjugating the antibodies to a light-generating moiety, administering the conjugate to a subject by, for example, injection, allowing the conjugate to localize to the site of the antigen, and imaging the conjugate.

Antibodies and antibody fragments have several advantages for use as entities in the present invention. By their nature, they constitute their own targeting moieties. Further, their size makes them amenable to conjugation with several types of light-generating moieties, including small fluorescent molecules and fluorescent and bioluminescent proteins, yet allows them to diffuse rapidly relative to, for example, cells or liposomes.

The light-generating moieties can be conjugated directly to the antibodies or fragments, or indirectly by using, for example, a fluorescent secondary antibody. Direct conjugation can be accomplished by standard chemical coupling of, for example, a fluorophore to the antibody or antibody fragment, or through genetic engineering. Chimeras, or fusion proteins can be constructed which contain an antibody or antibody fragment coupled to a fluorescent or bioluminescent protein. For example, Casadei, et al., describe a method of making a vector construct capable of expressing a fusion protein of aequorin and an antibody gene in mammalian cells.

Conjugates containing antibodies can be used in a number of applications of the present invention. For example, a labeled antibody directed against E-selection, which is expressed at sites of inflammation, can be used to localize the inflammation and to monitor the effects of putative anti-inflammatory agents.

Vector constructs by themselves can also constitute macromolecular entities applicable to the present invention. For example, a eukaryotic expression vector can be constructed which contains a therapeutic gene and a gene encoding a light-generating molecule under the control of a selected promoter (i.e. a promoter which is expressed in the cells targeted by the therapeutic gene). Expression of the light-generating molecule, assayed using methods of the present invention, can be used to determine the location and level of expression of the therapeutic gene. This approach may be particularly useful in cases where the expression of the therapeutic gene has no immediate phenotype in the treated individual or animal model.

3. Viruses. Another entity useful for certain aspects of the invention are viruses. As many viruses are pathogens which infect mammalian hosts, the viruses may be conjugated to a light-generating moiety and used to study the initial site and spread of infection. In addition, viruses labeled with a light-generating moiety may be used to screen for drugs which inhibit the infection or the spread of infection.

A virus may be labeled indirectly, either with an antibody conjugated to a light-generating moiety, or by, for example, biotinylating virions (e.g. by the method of Dhawan, et al.) and then exposing them to streptavidin linked to a detectable moiety, such as a fluorescent molecule.

Alternatively, virions may be labeled directly with a fluorophore like rhodamine, using, for example, the methods of Fan, et al. The virus can also be genetically engineered to express a light-generating protein. The genomes of certain viruses, such as herpes and vaccinia, are large enough to accommodate genes as large as the lux or luc genes used in experiments performed in support of the present invention.

Labeled virus can be used in animal models to localize and monitor the progression of infection, as well as to screen for drugs effective to inhibit the spread of infection. For example, while herpes virus infections are manifested as skin lesions, this virus can also cause herpes encephalitis. Such an infection can be localized and monitored using a virus labeled by any of the methods described above, and various antiviral agents can be tested for efficacy in central nervous system (CNS) infections.

4. particles. Particles, including beads, liposomes and the like, constitute another entity useful in the practice of the present invention. Due to their larger size, particles may be conjugated with a larger number of light-generating molecules than, for example, can small molecules. This results in a higher concentration of light emission, which can be detected using shorter exposures or through thicker layers of tissue. In addition, liposomes can be constructed to contain

an essentially pure targeting moiety, or ligand, such as an antigen or an antibody, on their surface. Further, the liposomes may be loaded with, for example, bioluminescent protein molecules, to relatively high concentrations (Campbell).

Furthermore, two types of liposomes may be targeted to the same cell type such that light is generated only when both are present. For example, one liposome may carry luciferase, while the other carries luciferin. The liposomes may carry targeting moieties, and the targeting moieties on the two liposomes may be the same or different. Viral proteins on infected cells can be used to identify infected tissues or organs. Cells of the immune system can be localized using a single or multiple cell surface markers.

The liposomes are preferably surface-coated, e.g., by incorporation of phospholipid—polyethyleneglycol conjugates, to extend blood circulation time and allow for greater targeting via the bloodstream. Liposomes of this type are well known.

5. Cells. Cells, both prokaryotic and eukaryotic, constitute another entity useful in the practice of the present invention. Like particles, cells can be loaded with relatively high concentrations of light-generating moieties, but have the advantage that the light-generating moieties can be provided by, for example, a heterologous genetic construct used to transfect the cells. In addition, cells can be selected that express "targeting moieties", or molecules effective to target them to desired locations within the subject. Alternatively, the cells can be transfected with a vector construct expressing an appropriate targeting moiety.

The cell type used depends on the application. For example, as is detailed below, bacterial cells, such as salmonella, can be used to study the infective process, and to evaluate the effects of drugs or therapeutic agents on the infective process with a high level of temporal and spatial resolution.

Bacterial cells constitute effective entities. For example, they can be easily transfected to express a high levels of a light-generating moiety, as well as high levels of a targeting protein. In addition, it is possible to obtain *E. coli* libraries containing bacteria expressing surface-bound antibodies which can be screened to identify a colony expressing an antibody against a selected antigen (Stratagene, La Jolla, Calif.). Bacteria from this colony can then be transformed with a second plasmid containing a gene for a light-generating protein, and transformants can be utilized in the methods of the present invention, as described above, to localize the antigen in a mammalian host.

Pathogenic bacteria can be conjugated to a light-generating moiety and used in an animal model to follow the infection process in vivo and to evaluate potential anti-infective drugs, such as new antibiotics, for their efficacy in inhibiting the infection. An example of this application is illustrated by experiments performed in support of the present invention and detailed below.

Eukaryotic cells are also useful as entities in aspects of the present invention. Appropriate expression vectors, containing desired regulatory elements, are commercially available. The vectors can be used to generate constructs capable of expressing desired light-generating proteins in a variety of eukaryotic cells, including primary culture cells, somatic cells, lymphatic cells, etc. The cells can be used in transient expression studies, or, in the case of cell lines, can be selected for stable transformants.

Expression of the light-generating protein in transformed cells can be regulated using any of a variety of selected promoters. For example, if the cells are to be used as light-emitting entities targeted to a site in the subject by an expressed ligand or receptor, a constitutively-active promoter, such as the CMV or SV40 promoter may be used. Cells transformed with such a construct can also be used to assay for compounds that inhibit light generation, for example, by killing the cells.

Alternatively, the transformed cells may be administered such they become uniformly distributed in the subject, and express the light-generating protein only under certain conditions, such as upon infection by a virus or stimulation by a cytokine. Promoters that respond to factors associated with these and other stimuli are known in the art. In a related aspect, inducible promoters, such as the Tet system (Gossen, et al.) can be used to transiently activate expression of the light-generating protein.

For example, CD4+ lymphatic cells can be transformed with a construct containing tat-responsive HIV LTR elements, and used as an assay for infection by HIV (Israel, et al.). Cells transformed with such a construct can be introduced into SCID-hu mice (McCune, et al.) and used as model for human HIV infection and AIDS.

Tumor cell lines transformed as above, for example, with a constitutively-active promoter, may be used to monitor the growth and metastasis of tumors. Transformed tumor cells may be injected into an animal model, allowed to form a tumor mass, and the size and metastasis of the tumor mass monitored during treatment with putative growth or metastasis inhibitors.

Tumor cells may also be generated from cells transformed with constructs containing regulatable promoters, whose activity is sensitive to various infective agents, or to therapeutic compounds.

6. Cell Transformation. Transformation methods for both prokaryotic cells and eukaryotic cells are well known in the art (Sambrook, et al.). Vectors containing the appropriate regulatory elements and multiple cloning sites are widely commercially available (e.g. Stratagene, La Jolla, Calif., Clontech, Palo Alto, Calif.).

IV. Transgenic Animals Containing Genes Encoding Light-Generating Proteins

In another aspect, the present invention includes transgenic animals containing a heterologous gene construct encoding a light-generating protein or complex of proteins. The construct is driven by a selected promoter, and can include, for example, various accessory proteins required for the functional expression of the light-generating protein, as well as selection markers and enhancer elements.

Activation of the promoter results in increased expression of the genes encoding the light-generating molecules and accessory proteins. Activation of the promoter is achieved by the interaction of a selected biocompatible entity, or parts of the entity, with the promoter elements. If the activation occurs only in a part of the animal, only cells in that part will express the light-generating protein.

For example, an interferon-inducible promoter, such as the promoter for 3'-5' poly-A synthetase, can be used to detect the infection of transgenic cells by a number of different RNA viruses.

In a related aspect, a promoter expressed in certain disease states can be used to mark affected areas in a transgenic animal, and expression of the light-generating moiety can be used to monitor the effects of treatments for the disease state. For example, E-selection is expressed at sites of inflammation in vivo (Pober, et al.). Accordingly, the E-selection promoter can be isolated and used to drive the expression of a luciferase gene.

It is also possible to use methods of the invention with tissue-specific promoters. This enables, for example, the

screening of compounds which are effective to inhibit pathogenic processes resulting in the degeneration of a particular organ or tissue in the body, and permits the tracking of cells (e.g. neurons) in, for example, a developing animal.

Many promoters which are applicable for use with the present invention are known in the art. In addition, methods are known for isolating promoters of cloned genes, using information from the gene's cDNA to isolate promoter-containing genomic DNA.

V. Imaging of Light-Emitting Conjugates

Light emitting conjugates that have localized to their intended sites in a subject may be imaged in a number of ways. Guidelines for such imaging, as well as specific examples, are described below.

A. Localization of Light-Emitting Conjugates

In the case of "targeted" entities, that is, entities which contain a targeting moiety—a molecule or feature designed to localize the entity within a subject or animal at a particular site or sites, localization refers to a state when an equilibrium between bound, "localized", and unbound, "free" entities within a subject has been essentially achieved. The rate at which such an equilibrium is achieved depends upon the route of administration. For example, a conjugate administered by intravenous injection to localize thrombi may achieve localization, or accumulation at the thrombi, within minutes of injection. On the other hand, a conjugate administered orally to localize an infection in the intestine may take hours to achieve localization.

Alternatively, localization may simply refer to the location of the entity within the subject or animal at selected time periods after the entity is administered. For example, in experiments detailed herein, Salmonella are administered (e.g., orally) and their spread is followed as a function of time. In this case, the entity can be "localized" immediately following the oral introduction, inasmuch as it marks the initial location of the administered bacteria, and its subsequent spread or recession (also "localization") may be followed by imaging.

In a related aspect, localization of, for example, injected tumors cells expressing a light-generating moiety, may consist of the cells colonizing a site within the animal and forming a tumor mass.

By way of another example, localization is achieved when an entity becomes distributed following administration. For example, in the case of a conjugate administered to measure the oxygen concentration in various organs throughout the subject or animal, the conjugate becomes "localized", or informative, when it has achieved an essentially steady-state of distribution in the subject or animal.

In all of the above cases, a reasonable estimate of the time to achieve localization may be made by one skilled in the art. Furthermore, the state of localization as a function of time may be followed by imaging the light-emitting conjugate according to the methods of the invention.

B. Photodetector Devices

An important aspect of the present invention is the selection of a photodetector device with a high enough sensitivity to enable the imaging of faint light from within a mammal in a reasonable amount of time, preferably less than about 30 minutes, and to use the signal from such a device to construct an image.

In cases where it is possible to use light-generating moieties which are extremely bright, and/or to detect light-emitting conjugates localized near the surface of the subject or animal being imaged, a pair of "night-vision" goggles or a standard high-sensitivity video camera, such as a Silicon Intensified Tube (SIT) camera (e.g. Hamamatsu Photonic Systems, Bridgewater, N.J.), may be used. More typically, however, a more sensitive method of light detection is required.

In extremely low light levels, such as those encountered in the practice of the present invention, the photon flux per unit area becomes so low that the scene being imaged no longer appears continuous. Instead, it is represented by individual photons which are both temporally and spatially distinct form one another. Viewed on a monitor, such an image appears as scintillating points of light, each representing a single detected photon.

By accumulating these detected photons in a digital image processor over time, an image can be acquired and constructed. In contrast to conventional cameras where the signal at each image point is assigned an intensity value, in photon counting imaging the amplitude of the signal carries no significance. The objective is to simply detect the presence of a signal (photon) and to count the occurrence of the signal with respect to its position over time.

At least two types of photodetector devices, described below, can detect individual photons and generate a signal which can be analyzed by an image processor.

1. Reduced-Noise Photodetection Devices. The first class constitutes devices which achieve sensitivity by reducing the background noise in the photon detector, as opposed to amplifying the photon signal. Noise is reduced primarily by cooling the detector array. The devices include charge coupled device (CCD) cameras referred to as "backthinned", cooled CCD cameras. In the more sensitive instruments, the cooling is achieved using, for example, liquid nitrogen, which brings the temperature of the CCD array to approximately −120° C. The "backthinned" refers to an ultra-thin backplate that reduces the path length that a photon follows to be detected, thereby increasing the quantum efficiency. A particularly sensitive backthinned cryogenic CCD camera is the "TECH 512", a series 200 camera available from Photometrics, Ltd. (Tucson, Ariz.).

2. Photon Amplification Devices. A second class of sensitive photodetectors includes devices which amplify photons before they hit the detection screen. This class includes CCD cameras with intensifiers, such as microchannel intensifiers. A microchannel intensifier typically contains a metal array of channels perpendicular to and co-extensive with the detection screen of the camera. The microchannel array is placed between the sample, subject, or animal to be imaged, and the camera. Most of the photons entering the channels of the array contact a side of a channel before exiting. A voltage applied across the array results in the release of many electrons from each photon collision. The electrons from such a collision exit their channel of origin in a "shotgun" pattern, and are detected by the camera.

Even greater sensitivity can be achieved by placing intensifying microchannel arrays in series, so that electrons generated in the first stage in turn result in an amplified signal of electrons at the second stage. Increases in sensitivity, however, are achieved at the expense of spatial resolution, which decreases with each additional stage of amplification.

An exemplary microchannel intensifier-based single-photon detection device is the C2400 series, available from Hamamatsu.

3. Image Processors. Signals generated by photodetector devices which count photons need to be processed by an image processor in order to construct an image which can be, for example, displayed on a monitor or printed on a video printer. Such image processors are typically sold as part of systems which include the sensitive photon-counting cam-

eras described above, and accordingly, are available from the same sources (e.g. Photometrics, Ltd., and Hamamatsu). Image processors from other vendors can also be used, but more effort is generally required to achieve a functional system.

The image processors are usually connected to a personal computer, such as an IBM-compatible PC or an Apple Macintosh (Apple Computer, Cupertino, Calif.), which may or may not be included as part of a purchased imaging system. Once the images are in the form of digital files, they can be manipulated by a variety of image processing programs (such as "ADOBE PHOTOSHOP", Adobe Systems, Adobe Systems, Mt. View, Calif.) and printed.

C. Immobilizing Subject in Detection Field of Device

1. Detection Field of Device. The detection field of the device is defined as the area from which consistent measurements of photon emission can be obtained. In the case of a camera using an optical lens, the detection field is simply the field of view accorded to the camera by the lens. Similarly, if the photodetector device is a pair of "night vision" goggles, the detection field is the field of view of the goggles.

Alternatively, the detection field may be a surface defined by the ends of fiber-optic cables arranged in a tightly-packed array. The array is constructed to maximize the area covered by the ends of the cables, as opposed to void space between cables, and placed in close proximity to the subject. For instance, a clear material such as plexiglass can be placed adjacent the subject, and the array fastened adjacent the clear material, opposite from the subject.

The fiber-optic cable ends opposite the array can be connected directly to the detection or intensifying device, such as the input end of a microchannel intensifier, eliminating the need for a lens.

An advantage of this method is that scattering and/or loss of photons is reduced by eliminating a large part of the air space between the subject and the detector, and/or by eliminating the lens. Even a high-transmission lens, such as the 60 mm AF Nikkor macro lens used in experiments performed in support of the present invention, transmits only a fraction of the light reaching the front lens element.

With higher-intensity LGMs, photodiode arrays may be used to measure photon emission. A photodiode array can be incorporated into a relatively flexible sheet, enabling the practitioner to partially "wrap" the array around the subject. This approach also minimizes photon loss, and in addition, provides a means of obtaining three-dimensional images of the bioluminescence.

Other approaches may be used to generate three-dimensional images, including multiple detectors placed around the subject or a scanning detector or detectors.

It will be understood that the entire animal or subject need not necessarily be in the detection field of the photodetection device. For example, if one is measuring a light-emitting conjugate known to be localized in a particular region of the subject, only light from that region, and a sufficient surrounding "dark" zone, need be measured to obtain the desired information.

2. Immobilizing the Subject. In those cases where it is desired to generate a two-dimensional or three-dimensional image of the subject, the subject may be immobilized in the detection field of the photodetection devices during the period that photon emission is being measured. If the signal is sufficiently bright that an image can be constructed from photon emission measured in less than about 20 milliseconds, and the subject is not particularly agitated, no special immobilization precautions may be required, except to insure that the subject is in the field of the detection device at the start of the measuring period.

If, on the other hand, the photon emission measurement takes longer than about 20 msec, and the subject is agitated, precautions to insure immobilization of the subject during photon emission measurement, commensurate with the degree of agitation of the subject, need to be considered to preserve the spatial information in the constructed image. For example, in a case where the subject is a person and photon emission measurement time is on the order of a few seconds, the subject may simply be asked to remain as still as possible during photon emission measurement (imaging). On the other hand, if the subject is an animal, such as a mouse, the subject can be immobilized using, for example, an anesthetic or a mechanical restraining device.

A variety of restraining devices may be constructed. For example, a restraining device effective to immobilize a mouse for tens of seconds to minutes may be built by fastening a plexiglass sheet over a foam cushion. The cushion has an indentation for the animal's head at one end. The animal is placed under the plexiglass such that its head is over the indentation, allowing it to breathe freely, yet the movement of its body is constrained by the foam cushion.

In cases where it is desired to measure only the total amount of light emanating from a subject or animal, the subject does not necessarily need to be immobilized, even for long periods of photon emission measurements. All that is required is that the subject be confined to the detection field of the photodetector during imaging. It will be appreciated, however, that immobilizing the subject during such measuring may improve the consistency of results obtained, because the thickness of tissue through which detected photons pass will be more uniform from animal to animal.

D. Further Considerations During Imaging

1. Fluorescent Light-Generating Moieties. The visualization of fluorescent light-generating moieties requires an excitation light source, as well as a photodetector. Furthermore, it will be understood that the excitation light source is turned on during the measuring of photon emission from the light-generating moiety.

Appropriate selection of a fluorophore, placement of the light source and selection and placement of filters, all of which facilitate the construction of an informative image, are discussed above, in the section on fluorescent light-generating moieties.

2. High-Resolution Imaging. Photon scattering by tissue limits the resolution that can be obtained by imaging LGMs through a measurement of total photon emission. It will be understood that the present invention also includes embodiments in which the light-generation of LGMs is synchronized to an external source which can be focused at selected points within the subject, but which does not scatter significantly in tissue, allowing the construction of higher-resolution images. For example, a focused ultrasound signal can be used to scan, in three dimensions, the subject being imaged. Light-generation from areas which are in the focal point of the ultrasound can be resolved from other photon emission by a characteristic oscillation imparted to the light by the ultrasound (e.g. Houston, et al.)

E. Constructing an Image of Photon Emission

In cases where, due to an exceptionally bright light-generating moiety and/or localization of light-emitting conjugates near the surface of the subject, a pair of "night-vision" goggles or a high sensitivity video camera was used to obtain an image, the image is simply viewed or displayed on a video monitor. If desired, the signal from a video

camera can be diverted through an image processor, which can store individual video frames in memory for analysis or printing, and/or can digitize the images for analysis and printing on a computer.

Alternatively, if a photon counting approach is used, the measurement of photon emission generates an array of numbers, representing the number of photons detected at each pixel location, in the image processor. These numbers are used to generate an image, typically by normalizing the photon counts (either to a fixed, pre-selected value, or to the maximum number detected in any pixel) and converting the normalized number to a brightness (greyscale) or to a color (pseudocolor) that is displayed on a monitor. In a pseudocolor representation, typical color assignments are as follows. Pixels with zero photon counts are assigned black, low counts blue, and increasing counts colors of increasing wavelength, on up to red for the highest photon count values. The location of colors on the monitor represents the distribution of photon emission, and, accordingly, the location of light-emitting conjugates.

In order to provide a frame of reference for the conjugates, a greyscale image of the (still immobilized) subject from which photon emission was measured is typically constructed. Such an image may be constructed, for example, by opening a door to the imaging chamber, or box, in dim room light, and measuring reflected photons (typically for a fraction of the time it takes to measure photon emission). The greyscale image may be constructed either before measuring photon emission, or after.

The image of photon emission is typically superimposed on the greyscale image to produce a composite image of photon emission in relation to the subject.

If it desired to follow the localization and/or the signal from a light-emitting conjugate over time, for example, to record the effects of a treatment on the distribution and/or localization of a selected biocompatible moiety, the measurement of photon emission, or imaging can be repeated at selected time intervals to construct a series of images. The intervals can be as short as minutes, or as long as days or weeks.

VI. Analysis of Photon Emission Images

Images generated by methods and/or using compositions of the present invention may be analyzed by a variety of methods. They range from a simple visual examination, mental evaluation and/or printing of a hardcopy, to sophisticated digital image analysis. Interpretation of the information obtained from an analysis depends on the phenomenon under observation and the entity being used.

The following experiments illustrate one application of the present invention—tracking salmonella infection in live mice—and how images obtained using methods of the present invention can be analyzed.

VII. Imaging of Luminescent Salmonella in Living Mice

Experiments performed in support of the present invention characterize the distribution of *Salmonella typhimurium* infection in mice, the animal model of human typhoid. A mouse virulent *Salmonella typhimurium*strain, SL1344 (Hoiseth and Stocker, 1981), a non-invasive mutant of SL1344, BJ66 and a low virulence LT-2 strain of salmonella, LB5000 were each marked with a plasmid containing the lux operon, and used in experiments to localize salmonella infection in mice.

A. Constructions of Luminescent Salmonella

1. Salmonella Strains. Three strains of *Salmonella typhimurium* with differing virulence phenotypes, defined by oral and intra-peritoneal inoculations into mice, are selected for transformation.

The most virulent phenotype used herein is SL1344, a mouse strain originally obtained from a fatal infection of a calf (Hoiseth and Stocker 1981). Following oral inoculations of mice with this strain, bacteria are disseminated systematically via the lymphatic system resulting in colonization of the liver, spleen and bone marrow (Carter and Collins, 1974; see also reviews by Finlay, et al., 1989, and Hsu, 1989).

A non-invasive mutant of SL1344, BJ66, is also evaluated. Systemic infections in mice do not typically result from an oral inoculation with BJ66, but do result from intraperitoneal inoculations with this strain.

A low virulence LT-2 strain of salmonella, LB5000, is also examined. LT-2 stains are laboratory strains known to be of reduced or variable virulence for mice. LB5000 contains multiple auxotrophic mutations, is streptomycin resistant, and is cleared from mice following oral or intraperitoneal inoculations.

2. Transformation of Salmonella Strains with the lux Operon. The three strains are each transformed with a plasmid encoding the lux operon, as detailed in Example 1. The plasmid, obtained from the soil bacterium *Xenorhabdus luminescens* (Frackman, et al., 1990) confers on *E coli* the ability to emit photons through the expression of the two subunits of the heterodimeric luciferase and three accessory proteins, luxC, luxD and luxE.

Inclusion of luxC, luxD and luxE removes the necessity of providing the fatty aldehyde substrate, luciferin, to the luciferase-expressing cells. Because supplying the substrate to eukaryotic luciferase enzymes in an in vivo system such as described herein may prove difficult, the entire lux operon of *X. luminescens* is used. The operon also encodes the enzymes for the biosynthesis of the fatty aldehyde substrate.

X. luminescens luciferase, an alpha-beta heterodimeric mixed-function oxidase, catalyzes the oxidation of reduced flavin and long-chain aldehyde to oxidized flavin and the corresponding long-chain fatty acid. A fatty acid reductase complex is required for the generation and recycling of fatty acid to aldehyde, and an NAD(P)H:flavin oxidoreductase supplies the reduced flavin.

Optimal bioluminescence for *E. Coli* expressing the lux genes of *X. luminescens* is 37° C. (Szittner and Meighen, Xi, et al.). In contrast, luciferases from eukaryotic and other prokaryotic luminescent organisms typically have lower temperature optima (Campbell). The luciferase from *X. luminescens*, therefore, is well-suited for use as a marker for studies in animals.

The three strains are transformed by electroporation with the plasmid pGSL1, which contains the entire *X. luminescens* lux operon and confers resistance to ampicillin and carbenicillin on the salmonella (Frackman, et al., 1990). The *X. luminescens* lux operon contains the genes luxA, luxB, luxC, luxD and luxE (Frackman, et al., 1990). LuxA and B encode the two subunits of the heterodimeric luciferase. luxC and D encode the biosynthetic enzymes for the luciferase substrate and luxE is a regulatory gene. Inclusion of the genes for the biosynthesis of the substrate is a convenient means of providing substrate to luciferase, in contrast to supplying luciferin externally to the cells in culture or treating animals with the substrate.

B. Characterization of Transformed Salmonella In Vitro

1. Adherence and Invasive Properties. The adherence and invasive properties of the three salmonella strains containing the lux plasmid are compared in culture, to each other, and to their non-luminescent parental strains by the standard invasion assay as described by Finlay, et al., and detailed in Example 2.

In this assay, adherent and intracellular bacteria are quantified following incubation with an epithelial cell line and

peritoneal macrophages. The adherent and intracellular bacteria are detected and quantified by both the emission of photons from living cells, and colony forming units following lysis and plating the cell lysates on carbenicillin-containing plates.

The results of some of the assays are shown in FIGS. 2A through 2E and discussed in Examle 8. The phenotypes of the three strains transformed with the lux expressing plasmid are not significantly altered in comparison to the parental salmonella strains. In addition, there is a good correlation between the intensity of bioluminescence and the CFU from the HEp-2 cells and macrophages. The results show that luminescence, as an indicator of intracellular bacteria, is a rapid method for assaying the invasive properties of bacteria in culture.

BJ66 demonstrated reduced adherence to HEp-2 cells in comparison to SL1344, however, adherence of the two strains in primary cultures of murine peritoneal macrophages were comparable.

2. Light Emission. To evaluate the oxygen requirements of the system, 10 fold serial dilutions of bacteria are placed in glass capillary tubes and imaged, as detailed in Example 3.

FIG. 3 shows an image generated in one such experiment. Luminescence is only detected at the air-liquid interface, even in the tubes with small numbers of bacteria in air saturated medium (0.1 ml of air saturated buffer in 5 l results in a final O_2 concentration of 5 nM).

From these results, it is apparent that oxygen is likely a limiting factor for luminescence.

3. Light Transmission Through Animal Tissue. To determine the degree to which light penetrates animal tissue, light emitted from luminescent salmonella and transmitted through tissue is quantified using a scintillation counter, with the fast coincidence detector turned off to detect single photons. The background due to dark current of the photomultiplier tubes in this type of detection is significant, limiting the assay to samples with relatively strong photon emission.

Four tissue types of varying opacity are compared using this approach: muscle from chicken breast, skin from chicken breast, lamb kidney and renal medulla from lamb kidney. The number of photons that can be detected through tissue is approximately ten fold less than the controls without tissue.

4. Characterization of lux Salmonella in vivo.

a. Oral Administration. Oral inoculation is natural route of infection of mice or humans with salmonella and results in a more protracted course of disease. In order to study the progression of the salmonella infection following this route of inoculation, two strains of mice are infected with the three strains of salmonella. The results obtained using the resistant animals are discussed under the heading "Infection of Resistant Mice", below.

Balb/c mice are orally infected with suspensions of virulent SL1344lux, non-invasive BJ66lux and low virulence LB5000lux salmonella, as described in Example 5. Progression of the infection is followed by external imaging (Materials and Methods) over an 8 day period.

Representative images are shown in FIGS. 5A–F. At 24 hours post inoculation (p.i.), the bioluminescent signal is localized at a single focus in all infected animals (FIGS. 5A, 5C and 5E). Bioluminescence disappears in all animals infected with the low virulence LB5000lux by 7 days p.i. (FIG. 5B). Animals infected with the virulent SL1344lux, on the other hand, show virulent infection which often spreads over much of the abdominal cavity (FIG. 5F), though the time at which it begins to spread is highly variable from animal to animal. The infection by BJ66lux typically persists and remains localized at a single site (FIG. 5D).

b. I.P. Inoculation. To assess whether or not there is sufficient O_2 at the sites of salmonella replication for the oxidation of luciferin and subsequent luminescence (Campbell), photon emission is measured from the tissues of a respiring animal. Luminescent SL1344lux and LB5000lux are inoculated into the peritoneal cavities of two groups of Balb/c mice. 32 hours post inoculation (p.i.), the transmitted photons are imaged (FIG. 6).

In the mice infected with SL1344lux (left part of figure), transmitted photons are evident over a large surface, with foci of varying intensities visible. These images are indicative of a disseminated infection, and are consistent with widespread colonization of the viscera, possibly including the liver and mesenteric lymph nodes. In contrast, the distributions of transmitted photons from animals infected with the LB5000lux strain is very limited, indicating a limited infection.

The LB5000lux-infected mice remained healthy for several weeks p.i., while the SL1344lux-infected mice were nearly moribund and euthanized at 4 days p.i.

These experiments indicate that the level of O_2 in the blood and or tissues is adequate for bioluminescence of lux luciferase expressed by salmonella. Furthermore, the experiments are consistent with the invasive nature of the virulent strain SL1344 in comparison to the reduced virulent laboratory strain LB5000.

c. Infection of Resistant Mice. Mice which are heterozygous at the Ity locus ($Ity^{r/s}$) are resistant to systemic infections by *S. typhimurium* (Plant and Glynn, 1976). This locus, also called Bcg (Gros, et al., 1981) or Lsh (Bradley, 1977), regulates the pathogenic processes of certain intracellular pathogens, such as *Mycobacterium lepraemurium* (Forget, et al.), *M. Bovis* (Skamene, et al.) and *M. intracelluare* (Goto, et al.). An analogous genetic control of resistance and susceptibility to intracellular pathogens appears to be in humans as well (*M. tuberculosis* (Stead, Stead, et al.) and *M. leprae*).

The Ity locus is located on mouse chromosome 1 with two allelic forms, Ity^r (resistant, dominant) and Ity^s (sensitive, recessive). The gene encoded at the Ity locus apparently affects the ability of macrophages to disrupt the internalized pathogens (reviewed by Blackwell, et al.; see also Skamene, et al.) which in turn, affects the down stream function of the proposed macrophage-mediated transport of pathogens to other sites within the infected host. Balb\c mice are $Ity^{s/s}$ and 129 mice are $Ity^{r/r}$. The heterozygous Balb\c×129 mice ($Ity^{r/s}$) are used in experiments detailed herein.

Resistant 129×Balb/c ($Ity^{r/s}$) viable mice are infected by intragastric inoculation of 1×10^7 SL1344lux salmonella as detailed in Example 7. The animals are imaged daily for 8 days post injection (d.p.i.).

Results are shown in FIGS. 7A (day 1) and 7B (day 8). The luminescence, detected by external imaging, is apparent at 24 h p.i., and appeared to localized to a single site in all animals. The luminescent signal is present throughout the study period (up to 8 days p.i.). The intensity of the luminescence and the location of the luminescent source is somewhat variable over time within a mouse and also from mouse to mouse. The luminescent tissue in all infected animals is the cecum (see below) and the variability in localization, and possibly intensity, is most likely due fact that internal organs of rodents are not tightly fixed in position.

The apparent limited infection observed in these animals supports the interpretation that the Ity restriction blocks

macrophage transport. The persistence of this infection for 10 days, however, suggests that there is adherence to the intestine mucosa and prolonged shedding of bacteria in the feces of these animals, as evidenced by luminescent fecal pellets. These results indicate that the luminescent phenotype of the salmonella in vivo is retained over an 8 day duration in Ity restricted animals and that localization is possible following an oral inoculation.

d. Internal Imaging Following Oral Inoculation. In order to further localize the luminescent signal in the abdominal cavity, infected mice are imaged following laparotomy (Example 8). The predominant disease manifestation in all of the animals infected by the oral route is an enlarged cecum (FIGS. 8A–C). The "external" image (FIG. 8A) illustrates a focal luminescence, which is revealed in the post-laparotomy image (FIG. 8B) to be the cecum.

Injection of air into the intestine confirms the presence of bacteria in other regions of the digestive tract. Bacteria in the colon and rectum are likely expressing luciferase, but low oxygen concentrations are likely limiting light emission from these sites.

The images obtained from oral inoculation studies indicate that the luminescent signal, at 2 days p.i. and at 7 days p.i., localizes almost entirely to the cecum in each of the animals (Popesko, et al.) except those infected with LB5000lux. Luminescence is also apparent in the colon in some animals. By 7 days p.i., no luminescence is detectable in the LB5000lux-infected animals. The CFU present in the organs of these mice are determined at 2 and 5 d p.i.

In animals infected intragastrically with the invasive strain, SL1344lux, the luminescence in the cecum appears early and precedes a systemic infection. In contrast, infections with the non-invasive BJ66lux strain result in a persistent luminescence from the cecum that remains, in some animals, for the entire course of the study (8 days). By 8 days p.i., luminescence is detected over much of the abdominal surface, resembling the distribution of photons following an i.p. inoculation, in the SL1344lux infected mice.

Infections with SL1344lux appear to become systemic, as predicted, with progressively more photons being emitted from an increasing surface area. Luminescence appears to localize over the abdomen in infections with all strains with little detectable luminescence from outside this area. A large number of transmitted photons are localized as a single focus over the abdomen suggesting that even though the infection may be systemic, the greatest amount of replication may be in areas surrounding the intestine.

Localization of the luminescence over the cecum indicates that not only are there large numbers of organisms in this region of the intestine, but also suggests that the Salmonella associate with cells of the mucosa such that they can obtain sufficient oxygen for luminescence. Emission of photons from luciferase is oxygen dependent and the expected oxygen levels in the lumen of the cecum, or intestine in general, are below the levels required for luminescence. The luciferase reaction is not expected to be functional in the intestine unless the bacteria can obtain oxygen from cells of the intestinal epithelium.

Thus, the systemic infection seems to be related to the invasive phenotype and not to simply adherence to epithelial cells of the intestine. These experiments implicate the cecum in some role in the pathogenic process either in the carrier state or as a site of dissemination.

Monitoring the progression of infections to different tissues may greatly enhance the ability to understand these steps in the pathogenic process, and enable the screening for compounds effective to inhibit the pathogen at selected steps.

e. Internal Imaging Following I.P. Inoculation. Mice infected intraperitoneally with SL1344lux are imaged before and after laparotomy (Example 9). The results are shown in FIG. 9. The images demonstrate luminescence over a majority of the abdomen with multiple foci of transmitted photons. The cecum does not appear to contain luminescent salmonella. The results from these experiments indicate that all strains of salmonella have sufficient O_2 to be luminescent in the early phases of infection. However, entry of Salmonella into cells of the mucosa and subsequent systemic infection is likely limited to strains with the invasive phenotype, since systemic infections at later time points are only apparent in SL1344lux-infected mice.

f. Effects of Ciprofloxacin on Salmonella Infection. Experiments, detailed in Example 10, are performed to demonstrate that non-invasive imaging is useful for following the response of an infection to drugs. Mice are orally inoculated with SL1344lux and treated with 100 mg of ciprofloxacin, an antibiotic effective against salmonella infections. The mice are imaged at selected time periods following treatment, and the extent of infection is quantitated by measuring photon emission. Photon emission in treated mice is compared to values before the initiation of treatment, and to values from control mice that had been infected, but not treated. Results from one such experiment are shown in FIGS. 10A–E and discussed in Example 10. Infection is significantly reduced in mice treated with the antibiotic, compared both to the levels of pathogen at time zero in treated animals, and to levels of pathogen in control animals throughout the treatment period.

g. Effects of Carbenenicillin Selection. Ducluzeau, et al., demonstrated that treatment of animals with antibiotics facilitated colonization of the cecum with Salmonella. The mice in the present experiments are maintained on an antibiotic regime of intramuscular injections of carbenicillin for the purpose of selecting the Amp^r Salmonella containing the luciferase clone. This treatment may alter the course of the gastrointestinal infection, but the observation that Salmonella can associate with the cells lining the cecum indicates that oxygen is available for luminescence. This observation is notable, since the lumen of the cecum is commonly thought to be an anaerobic environment.

VIII. Applications

A. Determination of Oxygen Levels

The oxygen requirement for luminescence of luciferase evidenced in the experiments summarized above indicates that the present invention may be applicable as a method of determining spatial gradients of oxygen concentration in a subject. Luminescent bacteria have been used to measure oxygen levels in the range of 10–1 mM. The studies predict that 0.1 nM is the lower limit of detection (Campbell). The imaging methods described herein may be used for studying oxygen levels at various sites in living animals. For example, microorganisms that have been engineered to emit light in an O_2 or Ca^{2+}-dependent manner could be used as biosensors in a subject, much like luminescent bacteria are used in environmental analyses (Guzzo, et al., Korpela, et al., Jassim, et al.). The dynamic range of luminescence with respect to O_2 concentration is much broader and reaches lower O_2 concentrations than O_2 probes (Campbell). Moreover, light emission in proportion to O_2 concentration is linear over a range of 30 nM to 8 mM, and 9 mM O_2 is required for ½ maximal luminescence.

B. Localization of Tumor Cells

The growth and metastatic spread of tumors in a subject may be monitored using methods and compositions of the present invention. In particular, in cases where an individual

is diagnosed with a primary tumor, LECs directed against the cells of the tumor can be used to both define the boundaries of the tumor, and to determine whether cells from the primary tumor mass have migrated and colonized distal sites.

For example, LECs, such as liposomes containing antibodies directed against tumor antigens and loaded with LGMs, can be administered to a subject, allowed to bind to tumor cells in the subject, imaged, and the areas of photon emission can be correlated with areas of tumor cells.

In a related aspect, images utilizing tumor-localizing LECs, such as those described above, may be generated at selected time intervals to monitor tumor growth, progression and metastasis in a subject over time. Such monitoring may be useful to record results of anti-tumor therapy, or as part of a screen of putative therapeutic compounds useful in inhibiting tumor growth or metastasis.

Alternatively, tumor cells can be transformed with a luciferase construct under the control of a constitutively-active promoter, and used to induce luminescent tumors in animal models, as described above. Such animal models can be used for evaluating the effects of putative anti-tumor compounds.

C. Localization of Inflammation

In an analogous manner to that described above, compositions and methods of the present invention may be used to localize sites of inflammation, monitor inflammation over time, and/or screen for effective anti-inflammatory compounds. Molecules useful for targeting to sites of inflammation include the ELAN family of proteins, which bind to selections. An ELAN molecule can be incorporated as a targeting moiety on an entity of the present invention, and used to target inflammation sites.

Alternatively, an animal model for the study of putative anti-inflammatory substances can be made by making the animal transgenic for luciferase under the control of the E-selection promoter. Since E-selection is expressed at sites of inflammation, transgenic cells at sites of inflammation would express luciferase.

The system can be used to screen for anti-inflammatory substances. Inflammatory stimuli can be admimistered to control and experimental animals, and the effects of putative anti-inflammatory compounds evaluated by their effects on induced luminescence in treated animals relative to control animals.

D. Localization of Infection

As illustrated in experiments performed in support of the present invention and summarized above, LGCs may be effectively used to follow the course of infection of a subject by a pathogen. In experiments detailed herein, the LGCs are pathogenic cells (Salmonella) transformed to express luciferase. Such a system is ideally-suited to the study of infection, and the subsequent spread of infection, in animal models of human diseases. It provides the ability to monitor the progression of an infectious disease using sites of infection and disease progression rather than traditional systemic symptoms, such as fever, swelling, etc. in studies of pathogenesis.

Use of an external imaging method to monitor the efficacy of anti-infectives permits temporal and spatial evaluations in individual living animals, thereby reducing the number of animals needed for experiments pertaining to pathogenesis and/or the study anti-infective agents.

The following examples illustrate, but in no way are intended to limit the present invention.

MATERIALS AND METHODS

A. Cells

Salmonella strains SL1344 and LB5000 were obtained from B. A. D. Stocker (Stanford University; Hoiseth and Stocker 1981). Salmonella strain BJ66 was obtained from B. D. Jones (Stanford University).

HEp-2 cells were obtained from the American Type Culture Collection (ATCC; 12301 Parklawn Dr., Rockville Md.; Accession number CCL-23).

Murine peritoneal macrophages were obtained by peritoneal lavage of euthanized Balb/c mice with 7 ml of growth medium (Maximow, et al.).

B. Static Cultures

Low oxygen (static) cultures were prepared by inoculating 3 ml of LB Broth containing 100 mg/ml of carbenicillin with 6 µl of a bacterial suspension from a stationary phase culture, and growing the bacteria at 37° C. overnight in a stationary 7 ml culture tube.

C. Mice

Balb/c ($Ity^{s/s}$) mice were obtained from the Department of Oncology, Stanford University. 129×Balb/c ($Ity^{r/s}$) mice were obtained from the Stanford Transgenic Animal Facility (Stanford, Calif.). All animals were housed under identical conditions of photo period, feeding regime and temperature in the Stanford University Research Animal Facility (Stanford, Calif.).

Anesthesia was performed by injecting the animals intraperitoneally (i.p.) with 33 µg/kg body weight nembutal.

Euthanasia was performed by asphyxiation in CO_2 or cervical dislocation, following protocols recommended by the Stanford University Research Animal Facility. Cervical dislocation was used in experiments in which results may have been affected by physiological changes due to asphyxia.

Mice infected with lux-transformed salmonella were given daily intramuscular (i.m.) injections of carbenicillin (125 mg per kg body weight) to maintain selective pressure on the luminescent salmonella for retention of the Amp^r plasmid containing the lux operon.

D. Imaging

Animals or objects to be imaged were immobilized in a light-tight box containing a door and a charge-coupled device (CCD) camera with a two stage microchannel intensifier head (model C2400-40, Hamamatsu). The camera was attached, via cables leading out of the box, to an "ARGUS 50" image processor (Hamamatsu).

The ICCD system described above is capable of detecting single photons once a threshold of 10–30 photons is achieved. The signal to noise ratio of the system ranged from 2:1 to 1×10^4:1, depending on signal intensity.

Grey-scale images were obtained by opening the light box door in dim room light and integrating for 8–64 frames. The gain for the gray scale images was set to optimize the image—typically at 3000 volts on a scale of 0 to 10,000 volts.

Bioluminescence data were obtained in absence of external illumination. Exposure settings were as follows: the black level was set automatically by the camera/image processor, the gain was adjusted automatically by the intensifier controller, and the f-stop was set at 2.8. A 60 mm "AF NIKKOR" macro lens was used (Nikon Inc., Melville, N.Y.).

Bioluminescence images were generated by integrating photons for a selected period of time, typically 5 minutes.

Data are presented at the lowest bit range setting of 0–3 bits per pixel for all animals. For images of other objects, i.e. 24 well plates, where the resolution of the bioluminescent signals was not possible at a bit range of 0–3, the range was increased to a setting that permitted localization of bioluminescent signals, typically 1–7. Objects were imaged for shorter periods of time when additional information could not be obtained by imaging for five minutes.

External imaging refers to non-invasive imaging of animals. Internal imaging refers to imaging after a partial dissection of the animals, typically a laparotomy. Internal imaging is performed in selected animals to confirm the sources of photon emission localized by external imaging.

The bioluminescence image data are presented as a pseudo-color luminescence image representing the intensity of the detected photons. Six levels of intensity are typically used, ranging from blue (low intensity) to red (higher intensity).

To generate the figures presented herein, greyscale and bioluminescence images were superimposed, using the image processor, to form a composite image providing a spatial frame of reference.

The composite image was displayed on an RGB CRT (red, green, blue; cathode ray tube) monitor, and the monitor was photographed to produce hardcopies. Hardcopies were also generated by saving the image processor image as a digital file, transferring the file to a computer, and printing it on a color printer attached to the computer. Alternatively, hardcopies may be generated by printing the video signal directly using a video printer.

EXAMPLE 1

TRANSFORMATION OF SALMONELLA WITH pCGLS1 LUX PLASMID

Salmonella strains SL1344, BJ66 and LB5000 were transformed with pCGLS1, a pUC18-based vector encoding the lux operon from *Xenorhabdus luminescens* (Frackman, et al., 1990).

A. pCGLS1 plasmid

A schematic of the pCGLS1 plasmid is shown in FIGS. 1A, 1B and 1C. The plasmid was constructed by cloning an ~11 kb region encoding the lux genes from the soil bacterium *Xenorhabdus luminescens* (FIG. 1A; Frackman, et al., 1990) into the Bam HI site (FIG. 1B) of pUC18 (FIG. 1C; Clontech, Palo Alto, Calif.). The construction of the vector is described by Frackman, et al., (1990).

Restriction enzyme sites in FIG. 1A are represented as follows: Bs, Bst EII; C, Cla I; E, Eco RI; H, Hind III; M, Mlu I; S, Sca I; X, Xba I; B/Sa, Bam HI and Sau 3A junction. A sequence included in the multiple cloning site (MCS) is provided in FIG. 1B, with the Bam HI site indicated in bold type.

A graphical representation of a pUC18 vector with no insert is shown in FIG. 1C. Labeled elements include an ampicillin resistance gene (Ap), a lac Z gene (lac Z) and an *E. coli* origin of replication (Ori). The unmodified pUC18 vector is approximately 2.7 kb in size.

B. Transformation of Salmonella

Electrocompetent cells from salmonella strains SL1344, BJ66 and LB5000 were made using standard methods (Sambrook, et al.) and stored at –80° C. until just prior to use. Electroporation was performed as follows: 1 µl of the plasmid (0.2 µg/ml) was added to 40 µl of ice-cold electrocompetent cells suspended in 10% glycerol. The suspension was mixed gently for one minute, placed in a 1 mm gap electroporation cuvette and electroporated using a Bio-Rad Gene-Pulser (Bio-Rad Laboratories, Hercules, Calif.). The settings were 2.5 kvolts, 400 ohms and 25 µfarads.

Following a one hour agitated incubation in Luria Bertini (LB) broth at 37° C., the cells were plated on (LB) Agar containing 100 µg/ml carbenicillin and allowed to grow overnight.

Colonies were assayed for luminescence by visual inspection in a dark room. Five transformants were identified as having high levels of luminescence. Three of these, one each from the SL1344, BJ66 and LB5000 strains, were selected for subsequent experiments. They were termed SL1344lux, BJ66lux and LB5000lux, respectively.

EXAMPLE 2

INVASIVE POTENTIAL OF NORMAL AND TRANSFORMED SALMONELLA

The invasive potential of six strains of salmonella (SL1344lux, LB5000lux, BJ66lux, SL1344, LB5000 and BJ66) was determined using two types of bacterial adherence and entry assays. Colony-forming units (CFU) assays were performed essentially as previously described (Finlay, et al.) with modifications (Lee, et al.). Bioluminescence assays were performed essentially like the CFU assays, except that the number of cells was quantitated using bioluminescence, as opposed to CFUs.

Briefly, HEp-2 cells and primary murine peritoneal macrophages were seeded into 24-well tissue culture dishes at 1×10^5 cells per well in RPMI (Gibco/BRL, Grand Island, N.Y.) supplemented with 20 mM glutamine (Gibco/BRL) and 5% fetal calf serum (Hyclone, Logan, Utah). Twenty four hours (HEp-2) or seven days (macrophages) after cell seeding, bacteria from static cultures (see "Materials and Methods", above) were inoculated at 1×10^6 (multiplicity of infection (m.o.i.) of 10) or 1×10^7 (m.o.i. of 100, columns on right in FIGS. 2B–E) organisms per well and centrifuged onto the cell monolayer for 5 minutes at 1000 rpm (185×g) in a Beckman clinical centrifuge (Beckman Instruments, Columbia, Md.). The medium was replaced with RPMI medium (Gibco/BRL) either with (entry assay) or without (adherence assay) gentamicin (100 mg/ml). The co-cultures were incubated for a total of 3.5 hours at 35° C. in 5% CO_2.

Gentamicin in the incubation medium kills bacteria that had not been internalized by the HEp-2 cells, including those adhering to the surfaces of the HEp-2 cells. Accordingly, the signal in adherence assays (without gentamicin) represent both adherent and internalized bacteria, whereas the signal in entry assays (with gentamicin) represent only internalized bacteria.

Adherence and entry were assayed by imaging luminescent bacterial cells at three timepoints—1.5, 3.0 and 3.5 hours post inoculation. Prior to imaging at the first timepoint, the cell monolayer was washed three times with phosphate-buffered saline (PBS) to remove unattached bacteria and a fresh aliquot of RPMI medium was added. Luminescence was recorded using a 30 second exposure. Images at the second and third timepoints were obtained using a similar exposure, but without first washing the cells.

Data recorded at the last timepoint, displayed as pseudo-color luminescence images superimposed over gray scale images of the culture dish wells, are shown in FIG. 2A. The cell types, salmonella strains, and usage of gentamicin are indicated in the Figure. The data are also summarized as relative intensity of photon counts in the graphs in FIGS. 2B and 2D.

Following imaging at the 3.5 hour timepoint, the tissue culture cells were washed three times with PBS and lysed with 0.2% "TRITON X-100" in PBS. Adherent and/or intracellular bacteria, released by lysis, were plated on LB- or LB-carbenicillin agar plates and incubated for 18 h at 35° C. The number of bacteria released from each well was determined by counting the number of colony forming units (CFU, Finlay, et al., 1989, Lee, et al., 1990). These data are represented as the total bacterial colonies per ml recovered from co-culture after incubation for 3.5 h with or without gentamicin, and are summarized in the graphs in FIGS. 2C and 2E.

Data from both the bioluminescence and CFU assays indicate that (i) salmonella transformed with the lux genes have an infective potential similar to that of the parent lines, and (ii) luminescence detection and CFU determination yield comparable estimates for the invasive potential of the two salmonella strains in HEp-2 cells and macrophages. The ratio of bioluminescence to CFU was lower in macrophage cultures, possibly due to the subcellular compartment in which the salmonella enter macrophages.

EXAMPLE 3

IN VITRO LUMINESCENCE OF TRANSFORMED SALMONELLA

10 μl of four 10-fold serial dilutions (ranging from 10^6 cells to 10^3 cells per ml) of LB5000lux salmonella were placed in four 100 μl glass capillary tubes (Clay-Adams div. of Becton Dickinson, Parsippany, N.J.). The bacterial suspensions formed columns of fluid in the tubes, with pockets of air at both ends. One end of each tube was sealed with critoseal (Clay-Adams). The medium in which dilutions were made was saturated with O_2 through exposure to air.

The tubes were wrapped with clear plastic wrap and luminescence was determined by imaging for 30 seconds as described above. An exemplary image is shown in FIG. 3. Four tubes are pictured. They contained (from top to bottom) 10^6, 10^5, 10^4 and 10^5 salmonella cells/ml (10^4, 10^3, 10^2 and 10 cells/tube). Luminescence could be detected in suspensions containing as few as 10^4 cells/ml (100 cells). The luminescence is confined, however, to air/liquid interfaces, suggesting that the luminescence reaction requires relatively high levels of oxygen. Since many of the cells are presumably in the fluid column and not at the air/fluid interfaces, the data suggest that the luminescence in the capillary tubes shown in FIG. 3 arises from considerably fewer than the total number of cells in each tube.

EXAMPLE 4

IN VITRO DETECTION OF LUMINESCENCE THROUGH ANIMAL TISSUE

Micro test-tubes, constructed from glass capillary tubing with an internal diameter of 3.5 mm, containing serial dilutions of LB5000lux salmonella were prepared essentially as described in Example 3, above. In the present example, however, the bacterial suspensions contacted the sealed end of the tube and were exposed to air only at the upper end. The tubes were placed in a translucent plastic scintillation vial and surrounded by one of the following animal tissues: chicken breast muscle, chicken skin, lamb kidney or lamb renal medulla. All tissues were obtained from the meat department of a local supermarket (Safeway, Mountain View, Calif.).

A diagram of a vial containing a capillary tube surrounded by tissue is shown in FIG. 4. The vial 1 is approximately 1.4 cm in diameter and includes a cap 2. The vial is coated with an opaque material (i.e. black tape) along its upper portion 3. Animal tissue 4 is placed in the vial such that it extends from the bottom of the vial to just above the bottom edge of the opaque coating 3. The micro test-tube 5 is sealed at the bottom by a plug 7 (i.e. a crytoseal plug), and is centered radially in the vial, with the plugged end of the tube touching or in close proximity to the bottom of the vial. The bacterial suspension 6 extends approximately 1 cm upward from the bottom of the tube.

Photons emitted from vials with and without tissue, and with and without bacteria, were counted using a liquid scintillation counter (model 1219 Rackbeta, LKB/Wallac, Gaithersburg, Md.) with the fast coincidence discriminator disabled.

Controls without tissue were assayed by placing the bacterial suspension directly in the scintillation vial. All experiments were performed in triplicate.

In each experiment, the vials were counted two to three times, rotating the vial 90° between each count, to control for effects of possible tissue thickness inconsistency. No significant differences were detected.

The results are summarized in Table 2, below.

TABLE 2

TRANSMISSION OF PHOTONS THROUGH TISSUE

Sample	Chicken skin	Chicken muscle	Lamb kidney	Lamb medulla
Vial alone	2.1×10^4	1.3×10^4	1.0×10^4	1.0×10^4
Tissue alone	N.D.	1.5×10^4	9.4×10^3	8.5×10^3
Tissue and LB5000 lux*	2.7×10^5	2.3×10^5	1.6×10^4	1.5×10^5
LB5000 lux* alone	2.0×10^6	1.7×10^6	4.8×10^6	4.8×10^6

Counts are averages of triplicate measurements, tissue path length was 1 cm.
* — 1×10^7 cells.

The signal for 1×10^3 LB5000lux in kidney tissue was at or near background levels using the photomultiplier tubes (PMT) in the scintillation counter. The background in this type of detection is due to the dark current of the PMT and limits the studies to analysis of rather intense signals.

Bioluminescence from approximately 1×10^7 LB5000lux was detectable through 0.5 cm of avian muscle, skin ovine renal medulla and ovine kidney. These results indicate that bioluminescence from the labeled salmonella was detectable through animal tissues of variable opacity. Since oxygen was likely limited in the capillary tubes (as demonstrated in FIG. 3), it is likely that fewer numbers of bioluminescent salmonella could be detected through tissue than are indicated in this assay.

EXAMPLE 5

DETECTION OF ORALLY-ADMINISTERED LUX SALMONELLA IN BALB/C MICE

Balb/c mice were infected by oral feeding (Stocker, et al.) with a 50 μl suspension of 1×10^7 virulent SL1344lux, non-invasive BJ66lux and low virulence LB5000lux salmonella. The mice, 4–6 weeks of age at the time of infection, were imaged daily with 5 minute integration times (photon emission was measured for 5 minutes). Prior to imaging, the mice were anesthetized with 33 μg/kg body weight nembutal.

Representative images are shown in FIGS. 5A–F. At 24 hours post inoculation (p.i.), the bioluminescent signal local-

ized to a single focus in all infected animals (FIGS. 5A, 5C and 5E). Bioluminescence disappeared in all animals infected with the low virulence LB5000lux by 7 days p.i. (FIG. 5B). Animals infected with the virulent SL1344lux, on the other hand, showed virulent infection which often spread over much of the abdominal cavity (FIG. 5F). The spread of infection by BJ66lux was more variable, but the infection typically persisted and remained localized at the initial site (FIG. 5D).

EXAMPLE 6

DETECTION OF INFECTION FOLLOWING I.P. INOCULATION WITH A VIRULENT AND A LOW VIRULENCE STRAIN OF SALMONELLA

Balb/c mice were infected with either virulent (SL1344lux) or low virulence (LB5000lux) salmonella by intraperitoneal (i.p.) inoculations of 1×10^7 bacterial cells in a 100 μl suspension, without simultaneous injection of air.

At 32 hours post injection (p.i.), the mice were anesthetized and imaged as described above. The results are shown in FIG. 6. Widespread infection is evident in the two mice in the left part of the figure, infected with the virulent SL1344lux strain. In contrast, little, if any, luminescence is detected in the mice on the right, injected with the low virulence LB5000lux strain.

EXAMPLE 7

DETECTION OF SYSTEMIC INFECTION IN RESISTANT MICE FOLLOWING ORAL INOCULATION WITH SALMONELLA

Resistant 129×Balb/c ($Ity^{r/s}$) viable mice were infected by intragastric inoculation of 1×10^7 SL1344lux salmonella. The bacteria were introduced through an intra-gastric feeding tube while under anesthesia. The animals were imaged daily for 8 days post injection (d.p.i.).

Results are shown in FIGS. 7A and 7B. Mice, in triplicate, were infected and imaged daily for 8 days. Exemplary images for day 1 (FIG. 7A) and day 8 (FIG. 7B) are shown. These data indicate that mice resistant to systemic salmonella infection have a localized chronic infection in the cecum, but that the infection does not spread into the abdominal cavity.

EXAMPLE 8

POST-LAPAROTOMY IMAGING FOLLOWING ORAL INOCULATION WITH SALMONELLA

Laparotomy was performed following oral inoculation of salmonella to precisely localize the luminescent signal within the abdominal cavity, and to compare this localization with than obtained using non-invasive imaging. The animals were inoculated as described in Example 7. After a selected period of time, typically seven days, the mice were anesthetized and externally-imaged, as described above. An exemplary image is shown in FIG. 8A. After external imaging, the peritoneal cavity was opened and the animals were imaged again, as illustrated in FIG. 8B. In some instances the mice were imaged a third time, following injection of air into the lumen of the intestine both anterior and posterior to the cecum (C) (FIG. 8C). The mice were euthanized immediately after the final imaging.

EXAMPLE 9

POST-LAPAROTOMY IMAGING FOLLOWING I.P. INOCULATION WITH SALMONELLA

Balb/c mice were infected by intraperitoneal inoculation of 1×10^7 salmonella (SL1344lux) as described in Example 6. Exemplary images of one such animal are shown in FIGS. 9A, 9B and 9C.

At 24 hours post-injection (p.i.), the animal was anesthetized and imaged for five minutes (FIG. 9A). The peritoneal cavity was opened and the mouse was imaged again for five minutes (FIG. 9B). The cecum was pulled to the left side, and the animal was again imaged for five minutes (FIG. 9A).

The results demonstrate that the localization of infection sites obtained with non-invasive imaging correlates well with the sites as revealed upon opening the peritoneal cavity.

EXAMPLE 10

EFFECTS OF CIPROFLOXACIN TREATMENT ON BIOLUMINESCENCE FROM SL1344LUX SALMONELLA

Experimental and control groups of Balb/c mice were orally inoculated with SL1344lux. At 8 days p.i., mice in the experimental group were injected i.p. with 100 mg of ciprofloxacin hydrochloride (3 mg/kg body weight; Sigma Chemical Co., St. Louis, Mo.). Following treatment of the experimental group, animals from both groups were imaged (as above) at several intervals over a period of 5.5 h post treatment.

Representative images are shown in FIGS. 10B–E. FIGS. 10B and 10D show composite images of representative animals from the control and treated groups, respectively, immediately before initiation of treatment of the experimental group. FIGS. 10C and 10E show composite images of the same animals 5.5 hours after initiation of treatment. The total number of photons detected over the abdominal area were determined, normalized to the value at t=0, and plotted in FIG. 10A with respect to time post-treatment.

The data demonstrate that methods and compositions of the present invention can be used to evaluate the effects of drugs on the spread of infection in an animal model.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

It is claimed:

1. A noninvasive method for detecting the localization of an entity under study from within a mammalian subject, comprising

(a) administering to the subject a conjugate or transformed cell of the entity and a light-generating moiety or a transformed cell expressing the light-generating moiety,

(b) after a period of time in which the conjugate or transformed cell can achieve localization in the subject, immobilizing the subject within the detection field of a photodetector device,

(c) maintaining the subject in an immobilized condition,

(d) during said maintaining, measuring photon emission from the light-generating moiety, localized in the subject, with the photodetector device until an image of photon emission can be constructed, and

(e) detecting said image through an opaque tissue of said mammal.

2. The method of claim 1, which further includes repeating steps (b) through (e) at selected intervals,

wherein said repeating is effective to track the localization of the entity in the subject over time.

3. The method of claim 1, where said measuring is carried out with an intensified charge-coupled photodetector device.

4. The method of claim 1, for detecting the localization of infection by a pathogen in a mammalian subject, where said administering includes administering a conjugate or transformed cell that contains an infection-targeting moiety.

5. The method of claim 1, where said administering includes administering a conjugate that is a particle containing a light-generating moiety.

6. The method of claim 1, for detecting the localization of infection by a pathogen in an animal model, where the entity under study is the pathogen.

7. The method of claim 6, where the pathogen is Salmonella.

8. The method of claim 1, where the light-generating moiety is a light-generating protein.

9. The method of claim 8, where the protein is selected from the group consisting of luciferase, yellow fluorescent protein and ferredoxin IV.

10. The method of claim 8, where the entity under study is a transformed cell, and the light-generating moiety is a product of a heterologous gene expressed by the cell.

11. The method of claim 10, where expression of the heterologous gene is under the control of an activatable promoter.

12. A noninvasive method for detecting the level of an entity under study in a mammalian subject over time, comprising

(a) administering to the subject a conjugate of the entity and a light-generating moiety or a transformed cell expressing the light-generating moiety,

(b) placing the subject within the detection field of a photodetector device,

(c) maintaining the subject in the detection field of the device,

(d) during said maintaining, measuring photon emission from the light-generating moiety, in the subject, with the photodetector device, and

(e) repeating steps (b) through (d) at selected intervals, wherein said repeating is effective to detect changes in the level of the entity in the subject over time.

13. The method of claim 1, wherein step (d) involves measuring changes in the level of photon emission over time of said light-generating source.

14. The method of claim 1, wherein at least one light-generating source has the capability to emit light of a wavelength in the range of between 300 nm and 1100 nm.

15. The method of claim 14, wherein at least one light-generating source has the capability to emit light of a wavelength of 550 nm.

16. The method of claim 1, wherein said photodetector device comprises one or a plurality of photodetector device elements.

17. The method of claim 16, wherein one photodetector device element is a charge-coupled device (CCD) camera.

18. The method of claim 1, wherein said step (d) involves determining a measurement that is a function of a local condition of the light-generating moiety.

19. The method of claim 1, wherein said image in step (e) reflects a state of a condition selected from the group consisting of oxygen levels, determination of presence of tumor cells, localization of tumor cells, determination of presence of an inflammation, localization of an inflammation, determination of presence of an infection, and localization of an infection.

20. The method of claim 1, wherein said image in step (e) reflects the oxygen level.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,650,135
DATED : July 22, 1997
INVENTOR(S) : Christopher H. Contag; Pamela R. Contag; David A. Benaron

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 34, line 7, delete "1" and substitute therefor --12--.

Column 34, line 10, delete "1" and substitute therefor --12--.

Column 34, line 15, delete "1" and substitute therefor --12--.

Column 34, line 20, delete "1" and substitute therefor --12--.

Column 34, line 23, delete "1" and substitute therefor --12--.

Column 34, line 30, delete "1" and substitute therefor --12--.

Signed and Sealed this

Twenty-sixth Day of October, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO : 5,650,135
DATED : July 22, 1997
INVENTOR(S) : Christopher H. Contag; Pamela R. Contag; David A. Benaron

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 34, line 16, delete "1" and substitute therefor --12--.

Column 34, line 21, delete "1" and substitute therefor --12--.

Column 34, line 24, delete "1" and substitute therefor --12--.

Column 34, line 31, delete "1" and substitute therefor —12--.

Signed and Sealed this

Twenty-seventh Day of June, 2000

Attest:

Attesting Officer

Q. TODD DICKINSON

Director of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,650,135
DATED : July 22, 1997
INVENTOR(S) : Christopher H. Contag; Pamela R. Contag; David A. Benaron

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In column 1, at line 4, please insert:

-- This work was supported in part by grants from the Office of Naval Research (N-00014-91-C-0170), the National Institutes of Health (PHS M01-RR-00070-30/1 and RR-00081) and the United States Public Health Service. Accordingly, the United States Government has certain rights in this invention. --

Signed and Sealed this

Twenty-eighth Day of November, 2000

Attest:

Q. TODD DICKINSON

Attesting Officer

Director of Patents and Trademarks

US006217847B1

(12) **United States Patent**
Contag et al.

(10) **Patent No.: US 6,217,847 B1**
(45) **Date of Patent: Apr. 17, 2001**

(54) **NON-INVASIVE LOCALIZATION OF A LIGHT-EMITTING CONJUGATE IN A MAMMAL**

(75) Inventors: **Pamela R. Contag; Christopher H. Contag,** both of San Jose; **David A. Benaron,** Portola Valley, all of CA (US)

(73) Assignee: **The Board of Trustees of the Leland Stanford Junior University,** Palo Alto, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/233,507**

(22) Filed: **Jan. 19, 1999**

Related U.S. Application Data

(60) Division of application No. 08/602,396, filed on Feb. 16, 1996, now abandoned, which is a continuation-in-part of application No. 08/270,631, filed on Jul. 1, 1994, now Pat. No. 5,650,135.

(51) **Int. Cl.**[7] **A61K 49/00**

(52) **U.S. Cl.** **424/9.1**; 424/9.6; 424/9.61; 424/193.1; 436/800; 435/7.35; 435/7.9; 435/8; 435/172.3; 435/968; 435/69.1; 536/25.32; 536/24.1; 536/23.4

(58) **Field of Search** 424/9.1, 193.1, 424/258.1, 9.6, 9.61; 436/800; 536/25.32, 24.1, 23.4; 435/7.35, 7.9, 968, 172.3, 69.1, 8; 800/2

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Primary Examiner—Jennifer Graser
(74) *Attorney, Agent, or Firm*—Charles K. Sholtz; Peter J. Dehlinger

(57) **ABSTRACT**

Methods and compositions for detecting and localizing light originating from a mammal are disclosed. Also disclosed are methods for tracking light emission to selected regions, as well as for tracking entities within the mammal. In addition, animal models for disease states are disclosed, as are methods for localizing and tracking the progression of disease or a pathogen within the animal, and for screening putative therapeutic compounds effective to inhibit the disease or pathogen.

20 Claims, 24 Drawing Sheets

(18 of 24 Drawing Sheet(s) Filed in Color)

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FIG. IA

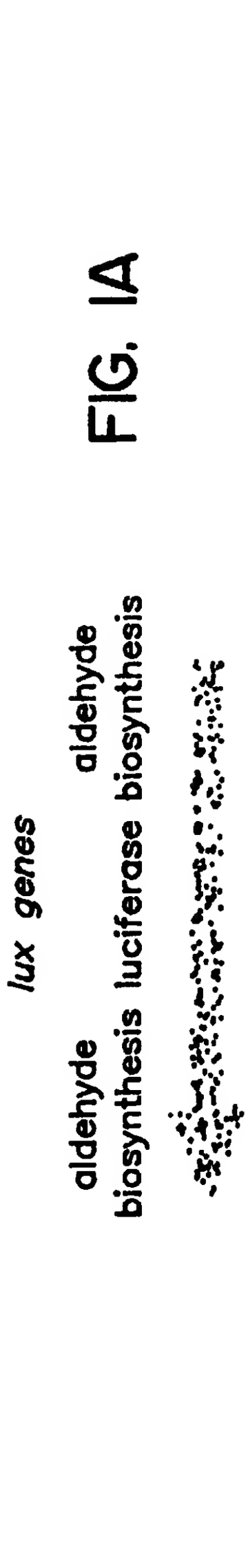
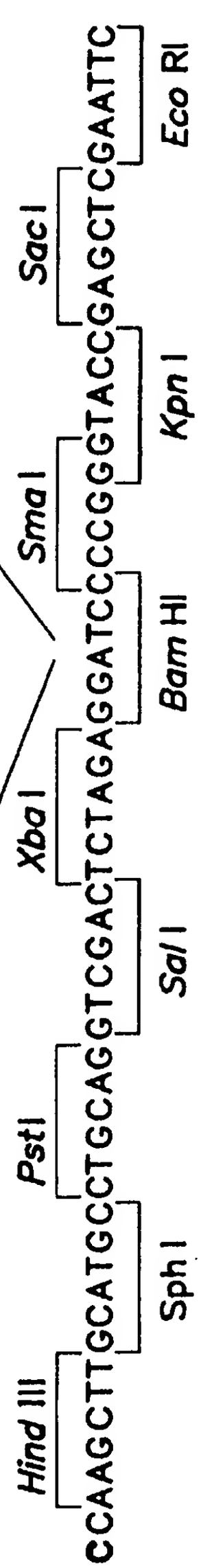

lux genes
aldehyde biosynthesis
luciferase
aldehyde biosynthesis
B/Sa E
HM H
BsCXH C
E
C S
B/Sa I

FIG. IB


Pst I
GCCTGCAGGTCGACTCTAGAGGATCCCCGG
Xba I
Sal I
Sac I
Sma I
Bam HI
Hind III
CCAAGCTTGCATGC
Sph I
Kpn I
GGTACCGAGCTCGAATTC
Eco RI
MCS
To Fig. 1C

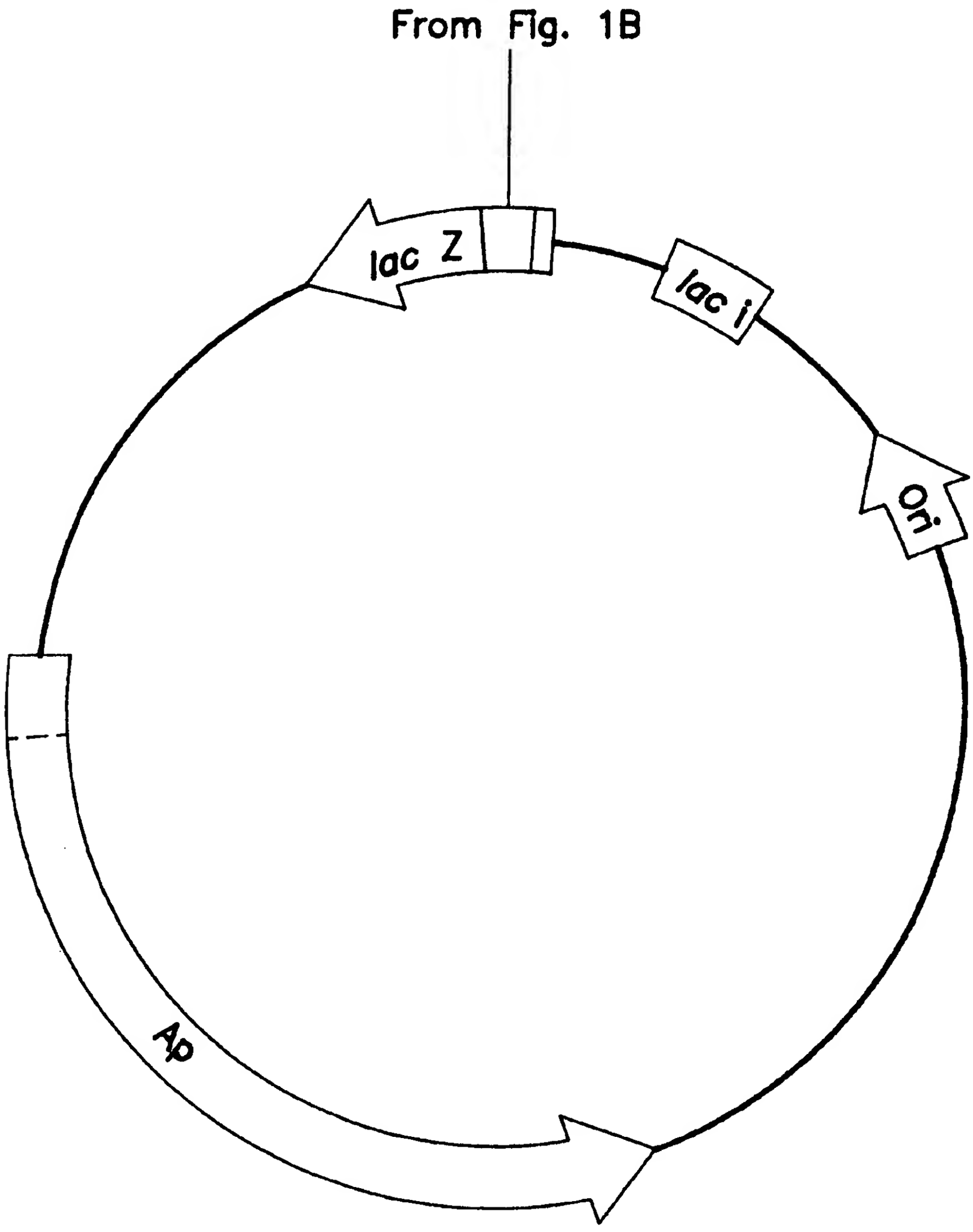


FIG. IC

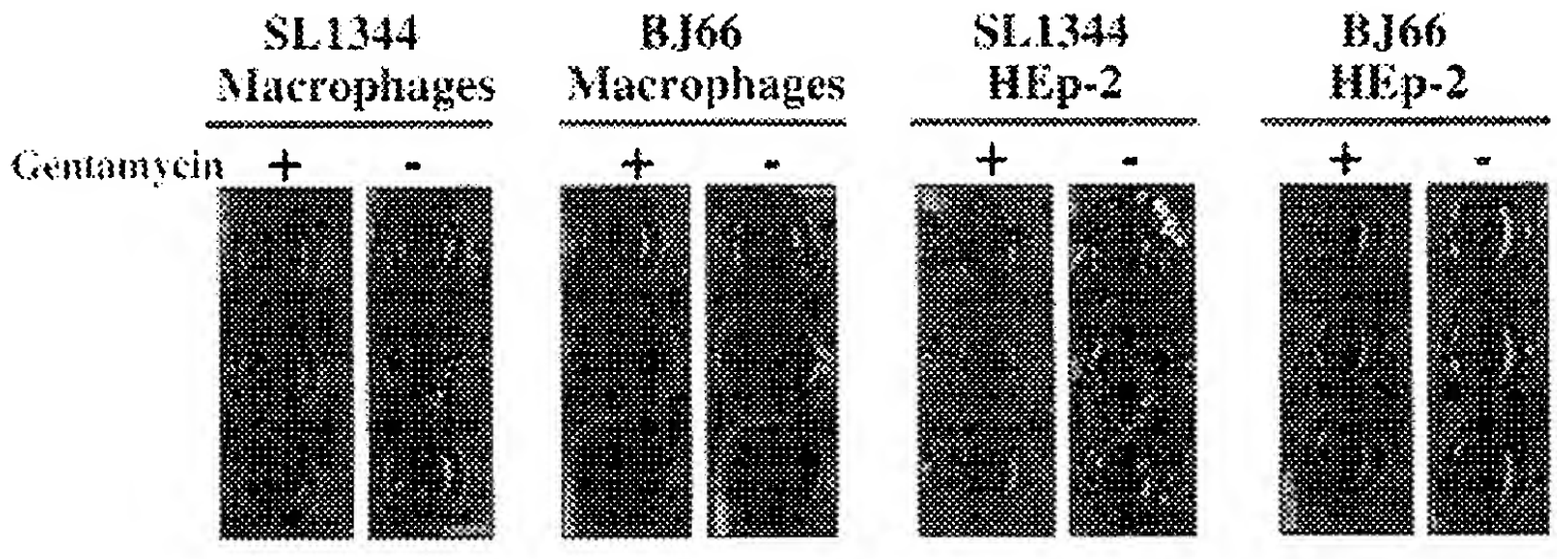

SL1344
Macrophages
BJ66
Macrophages
SL1344
HEp-2
BJ66
HEp-2
Gentamycin
+
-
+
-
+
-
+
-

FIG. 2A

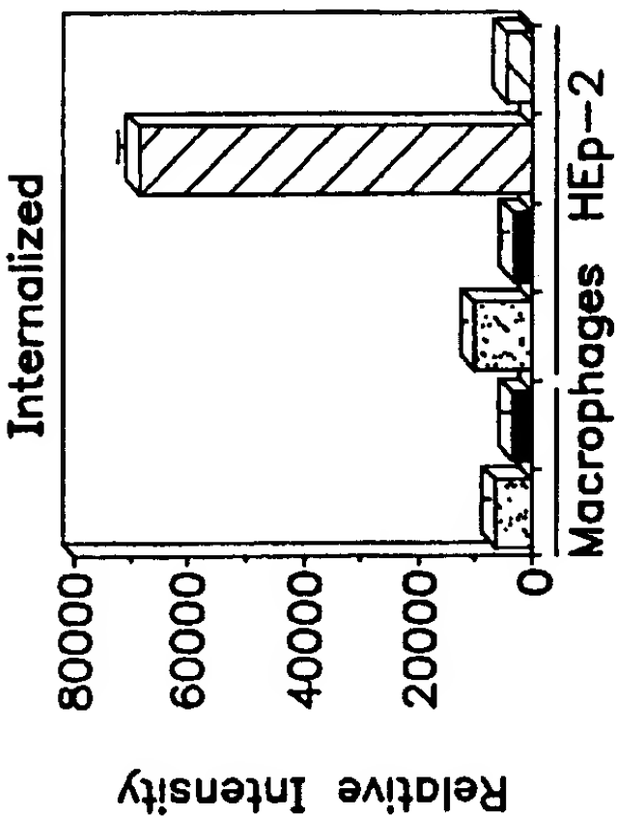


FIG. 2D

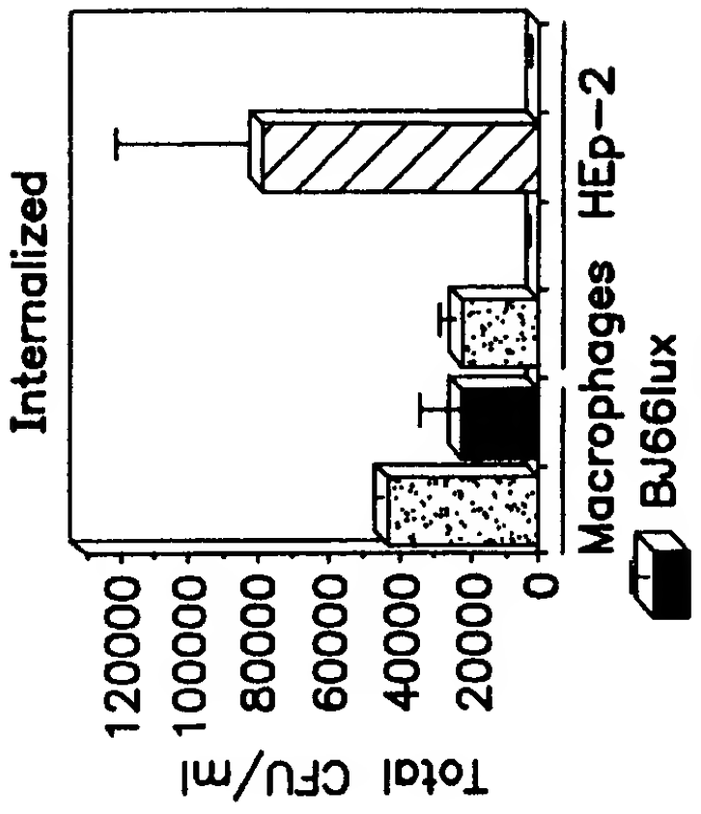


FIG. 2E

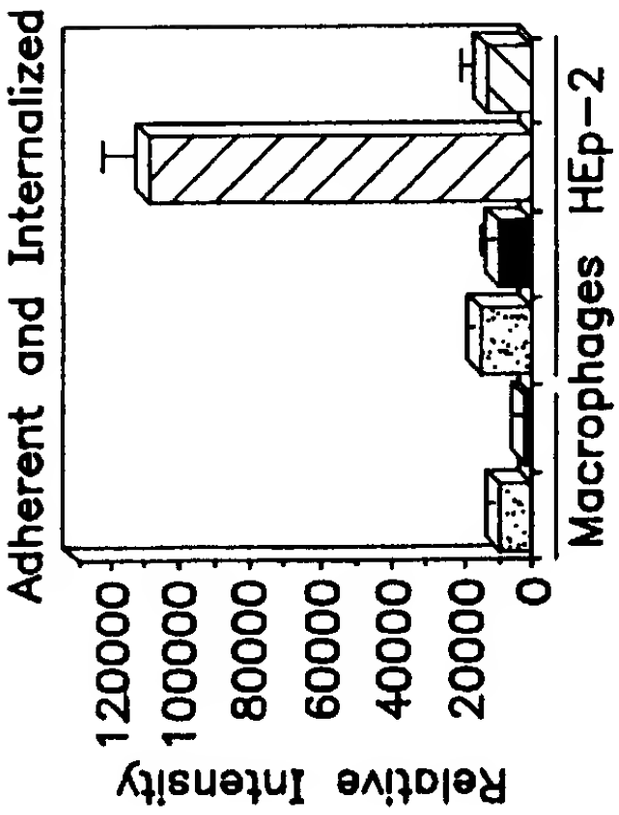


FIG. 2B

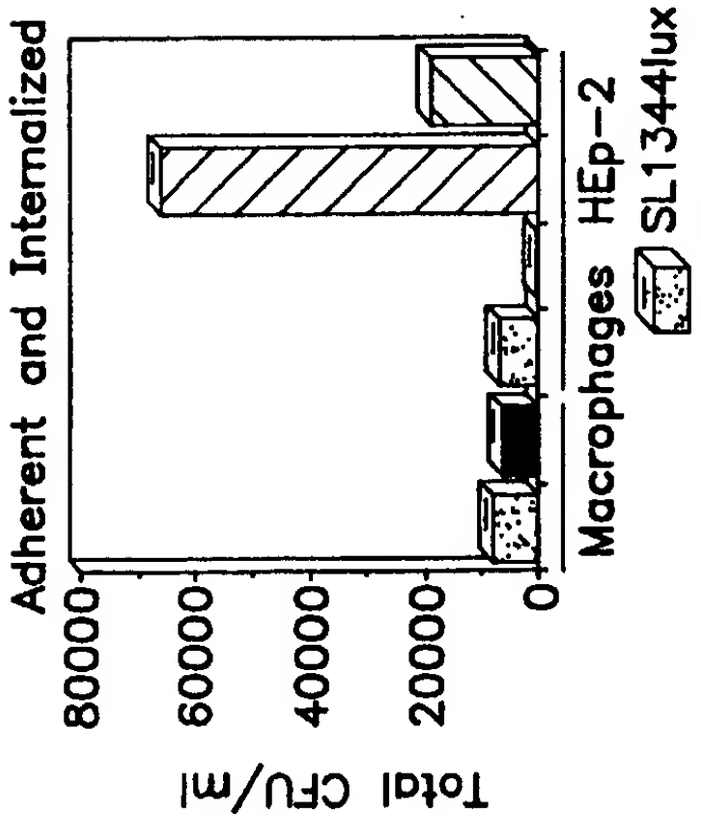


FIG. 2C

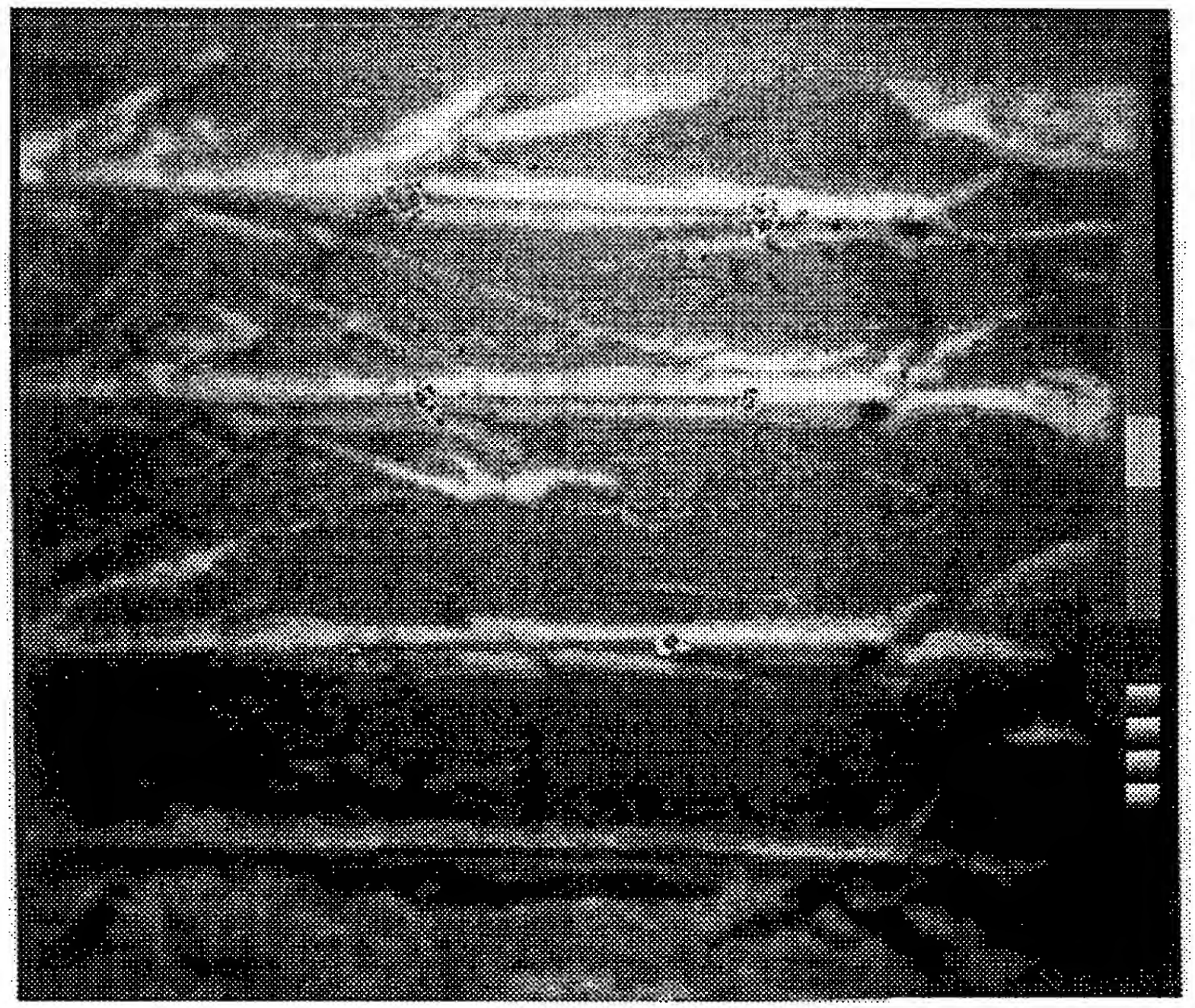
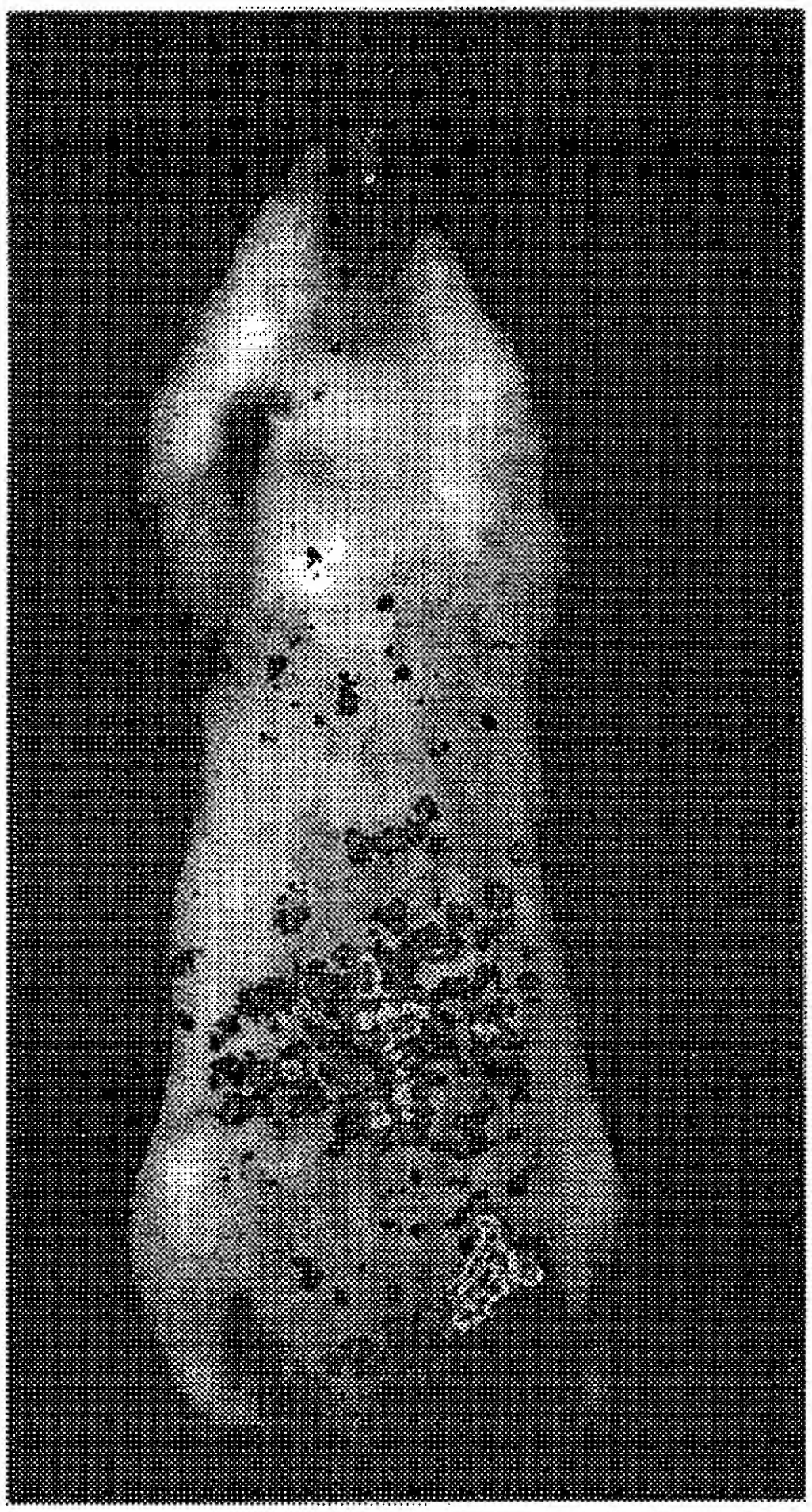


FIG. 3A



SL1344lux
(Intraperitoneal)

FIG. 3B

Effect of Human Blood on Signal from Bioluminescent *Salmonella*

Dilution	CFU/well	LB5000	Relative Bioluminescence	LB5000 and 30 µl blood	Relative Bioluminescence
1:100	512		199243		159497
1:200	256		187163		110081
1:400	128		170044		72234
1:800	64		154031		46273
1:1600	32		146934		17598
1:3200	16		112196		6731
1:6400	8		50302		320

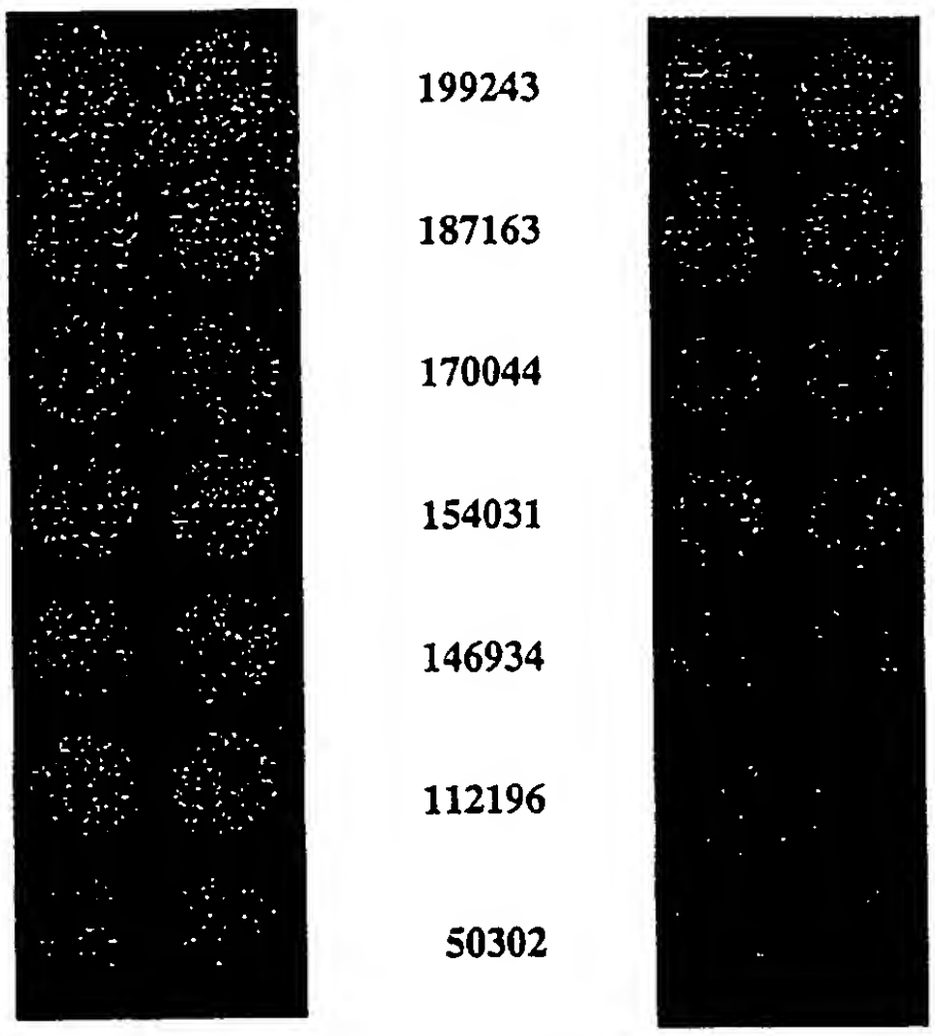


FIG. 4

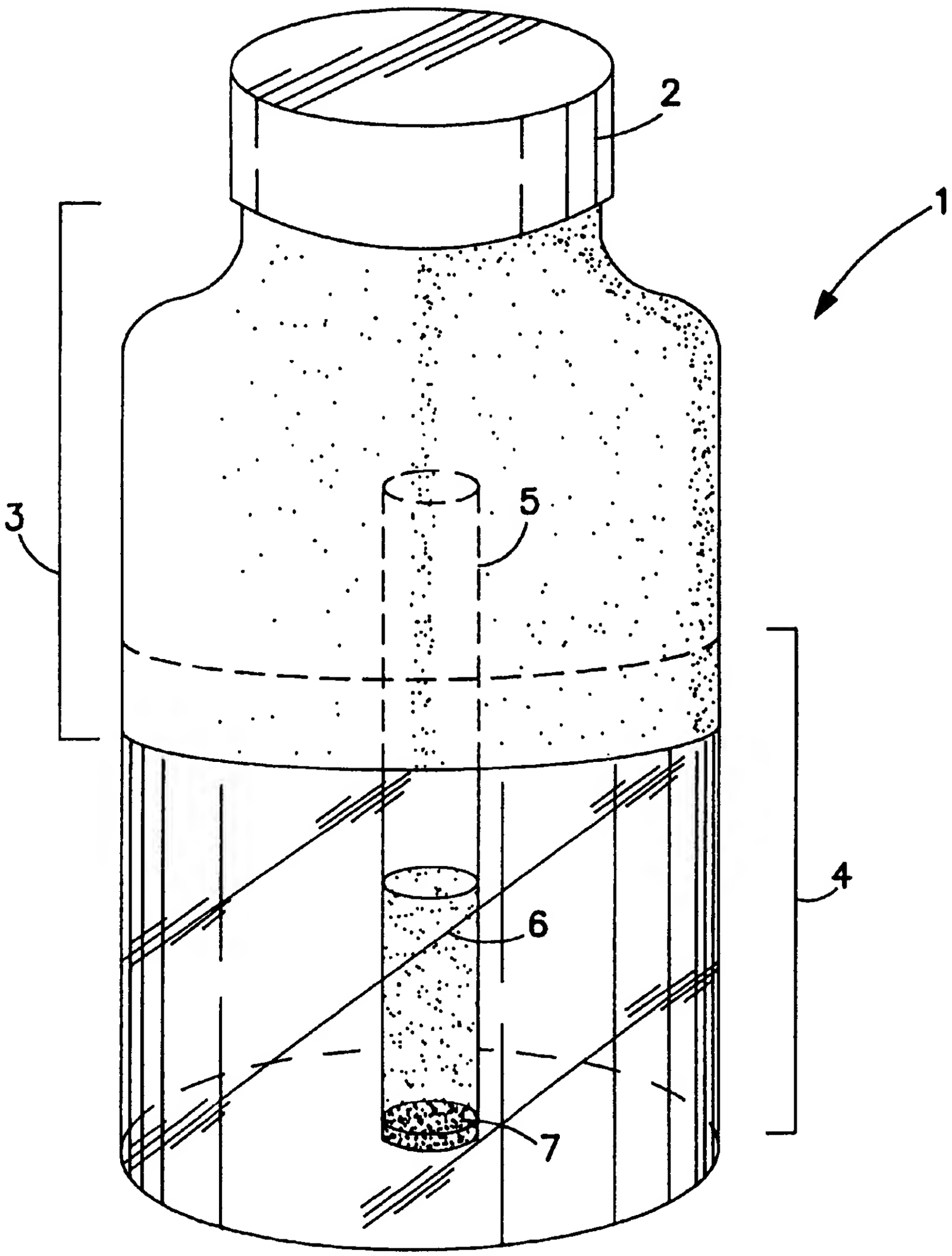
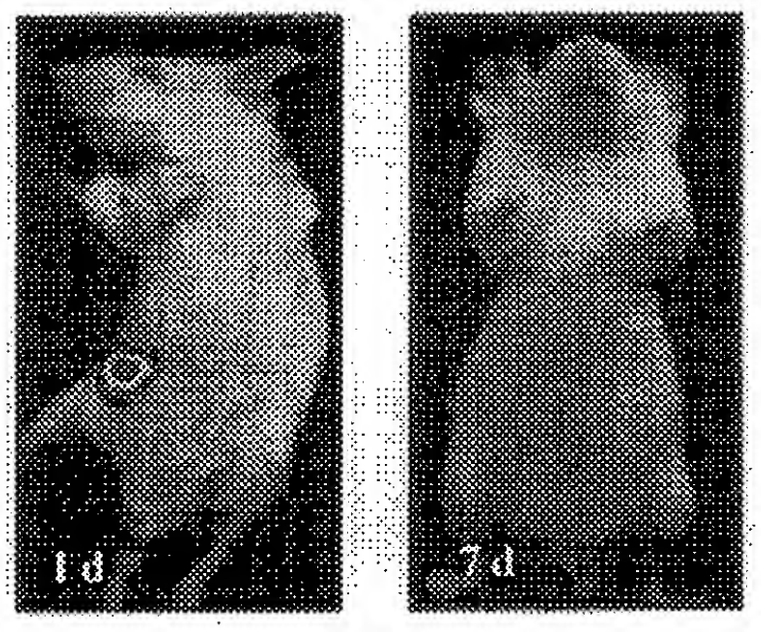
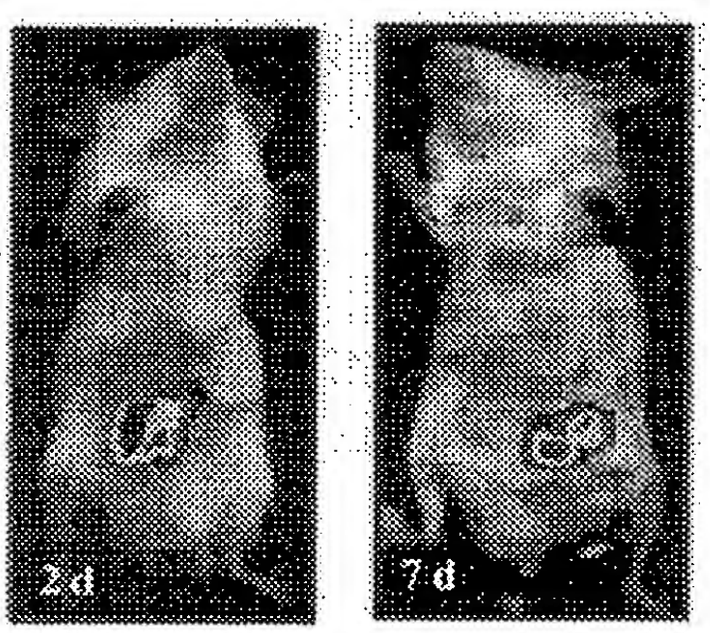


FIG. 5



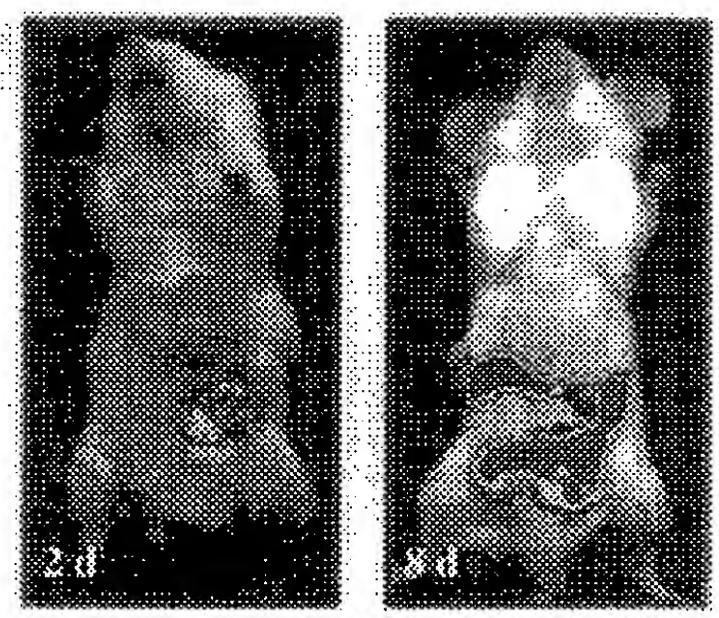
LB5000lux

FIG. 6A



BJ66lux

FIG. 6B



SL1344lux

FIG. 6C

FIG. 7

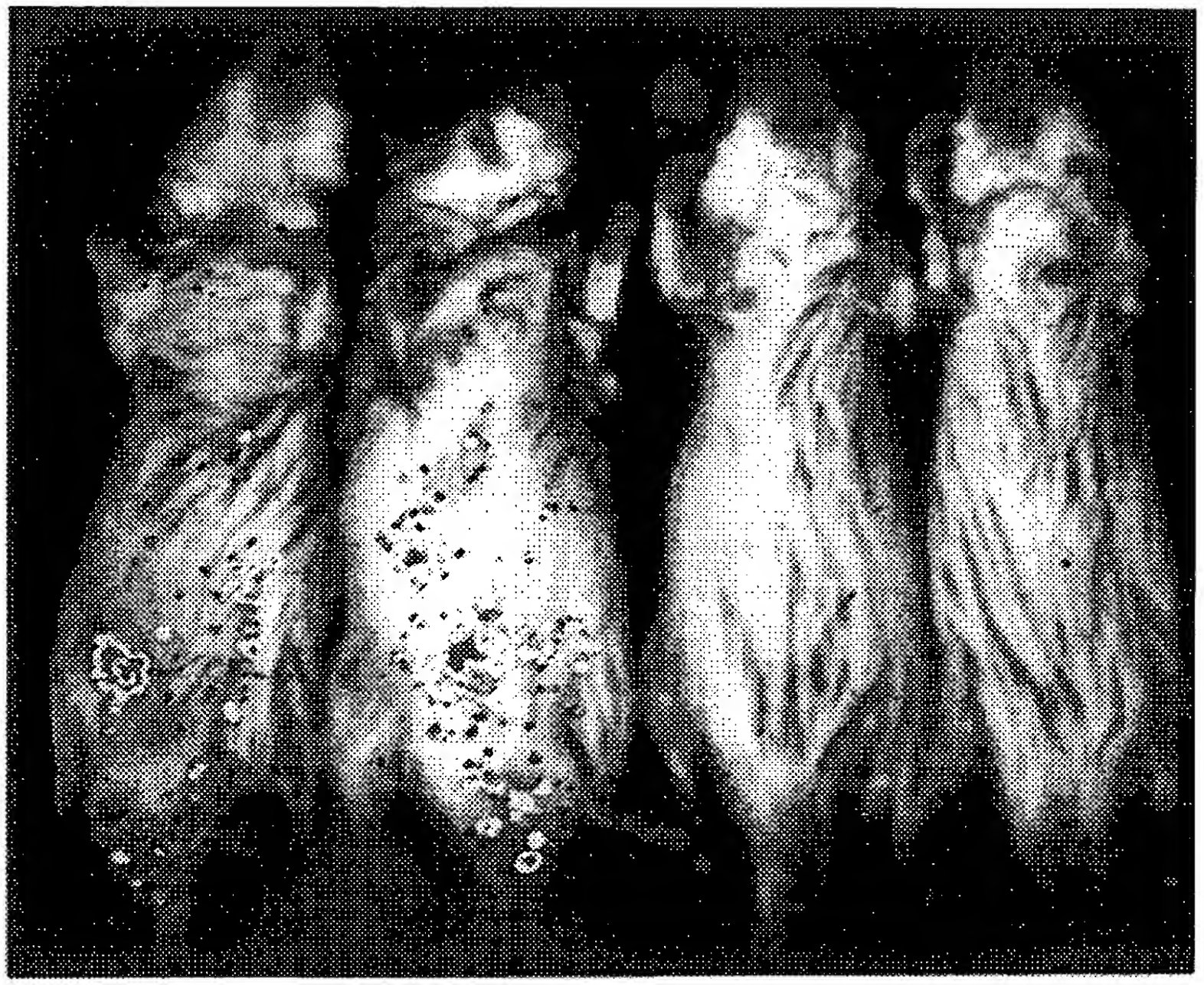


FIG. 8A

FIG. 8B

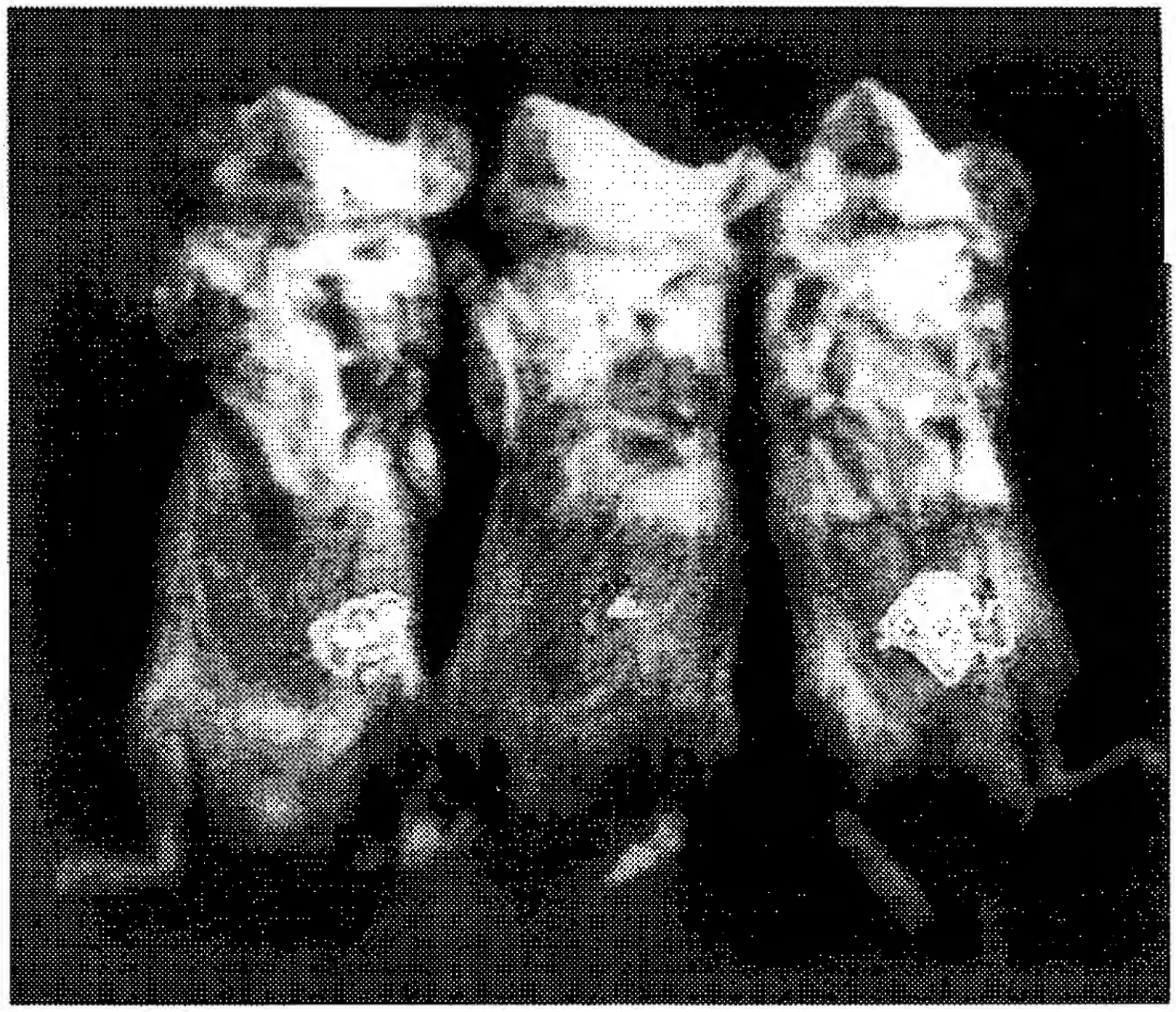
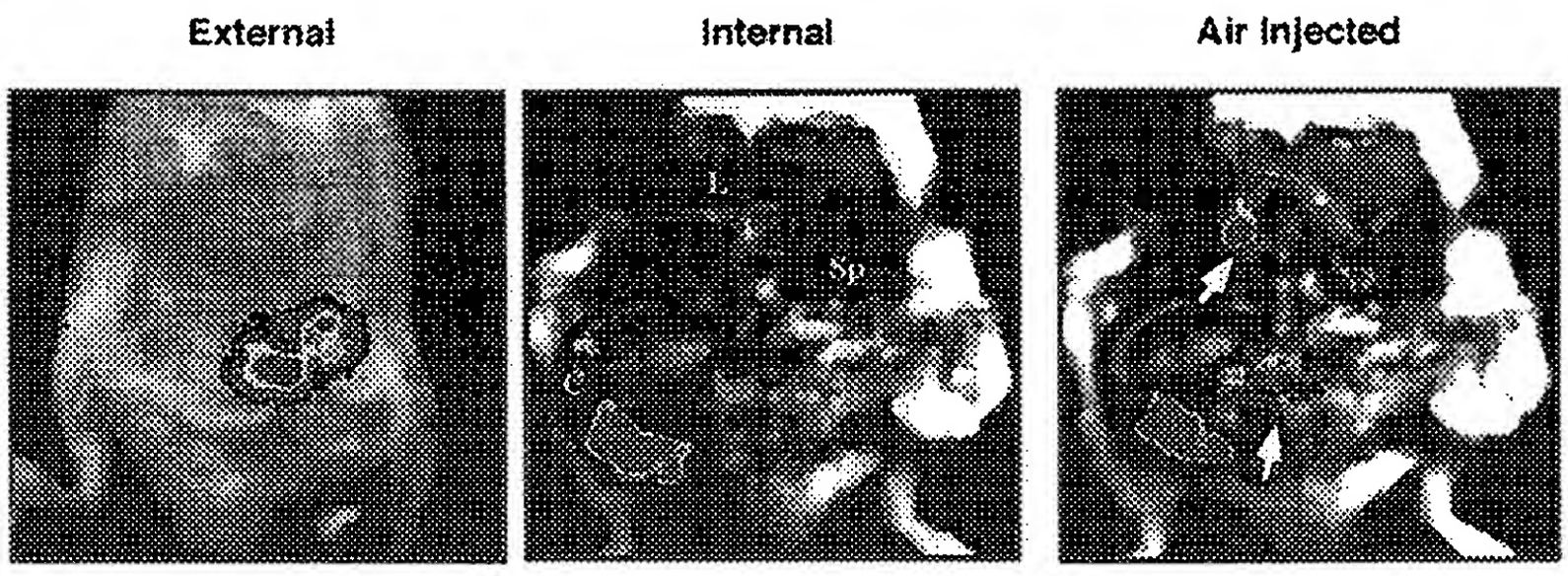


FIG. 9A FIG. 9B FIG. 9C



BJ66lux Infection (7 d p.i.)

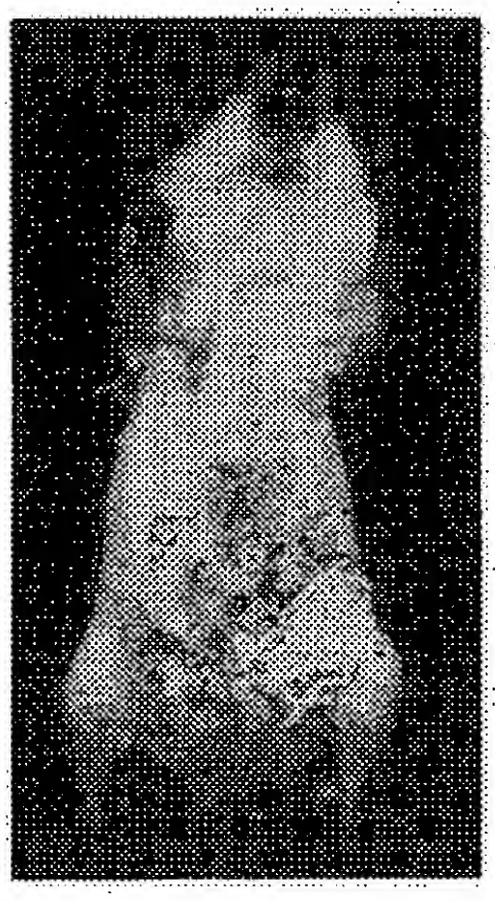


FIG. 10A

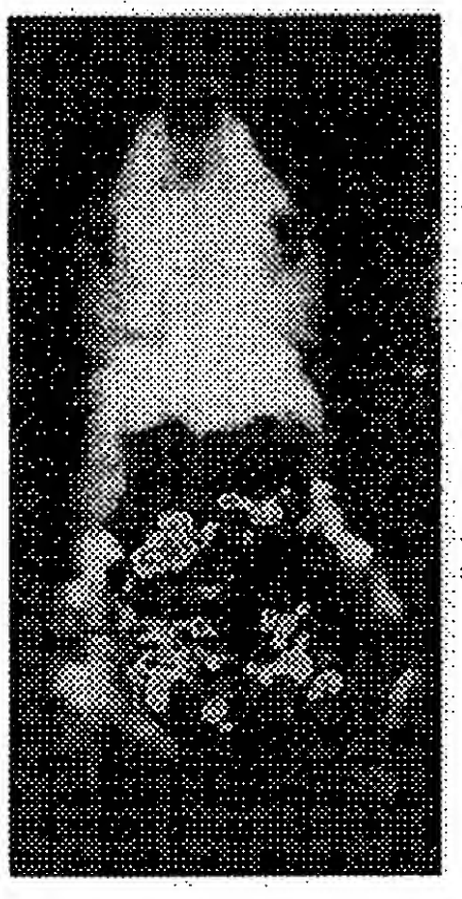


FIG. 10B

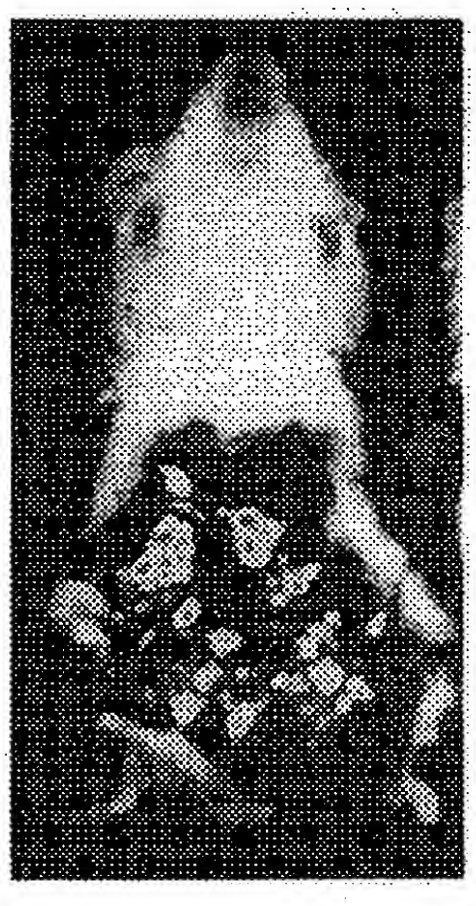


FIG. 10C

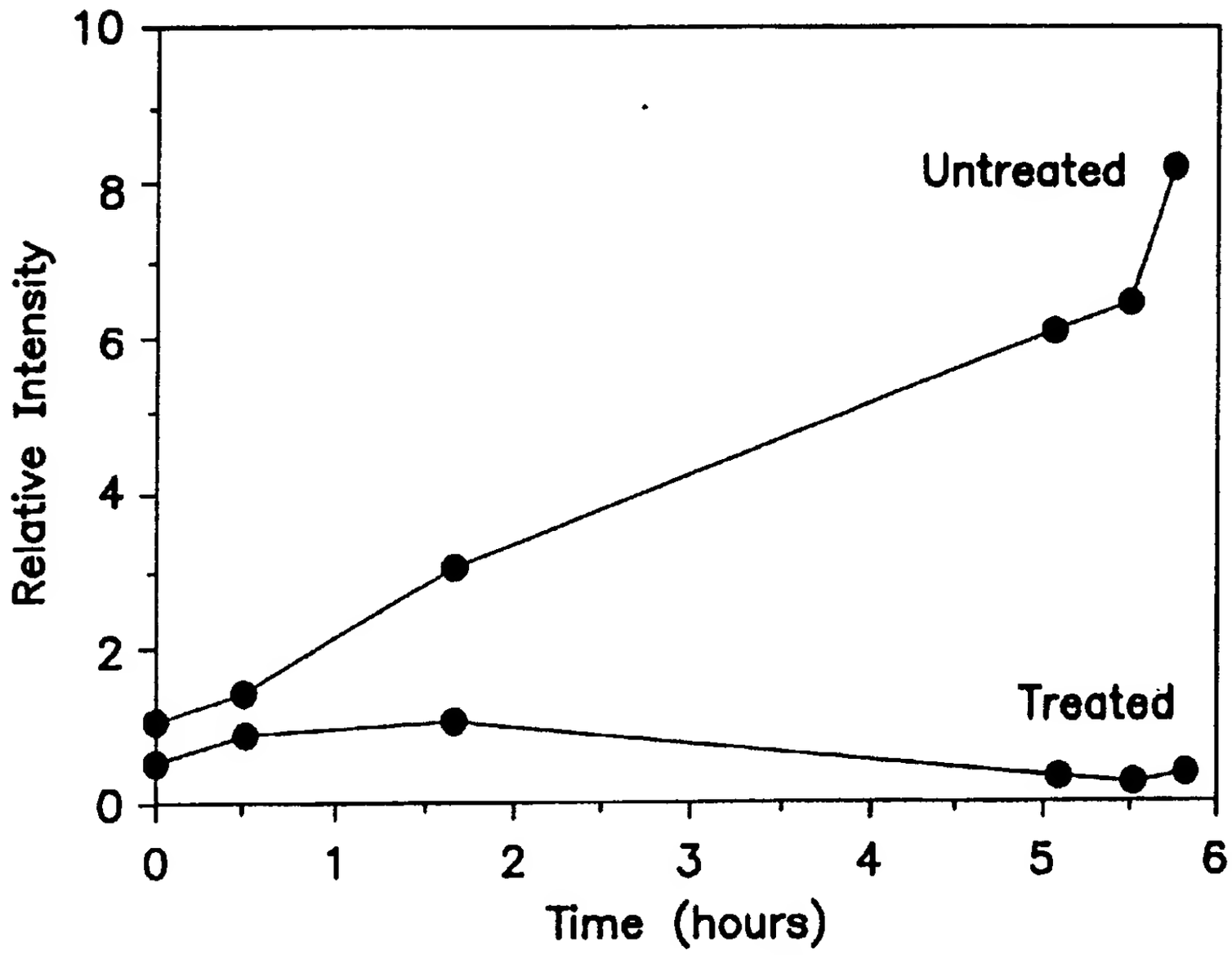

Relative Intensity
Time (hours)
Untreated
Treated
0
2
4
6
8
10
0
1
2
3
4
5
6

FIG. 11A

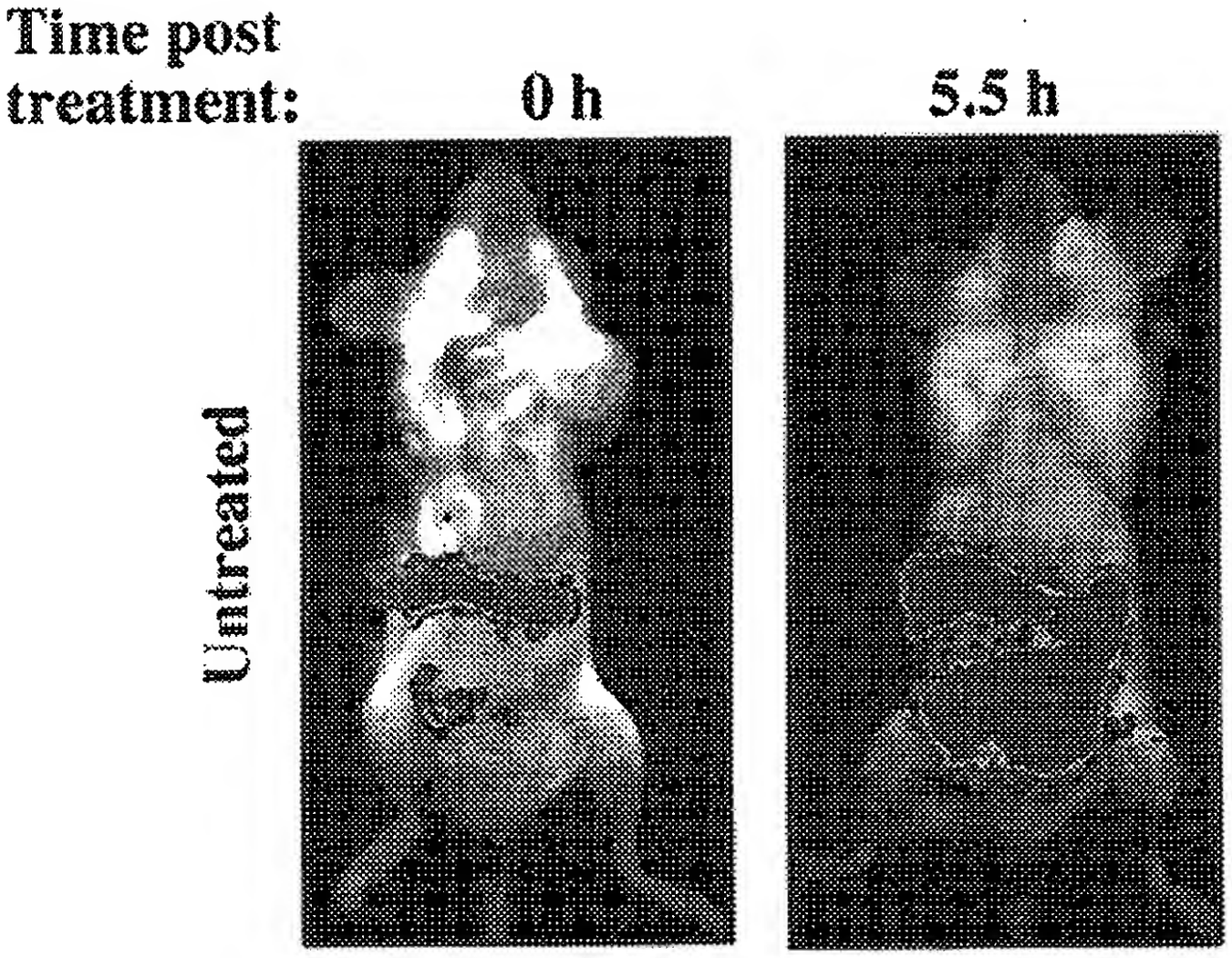


FIG. 11B FIG. 11C

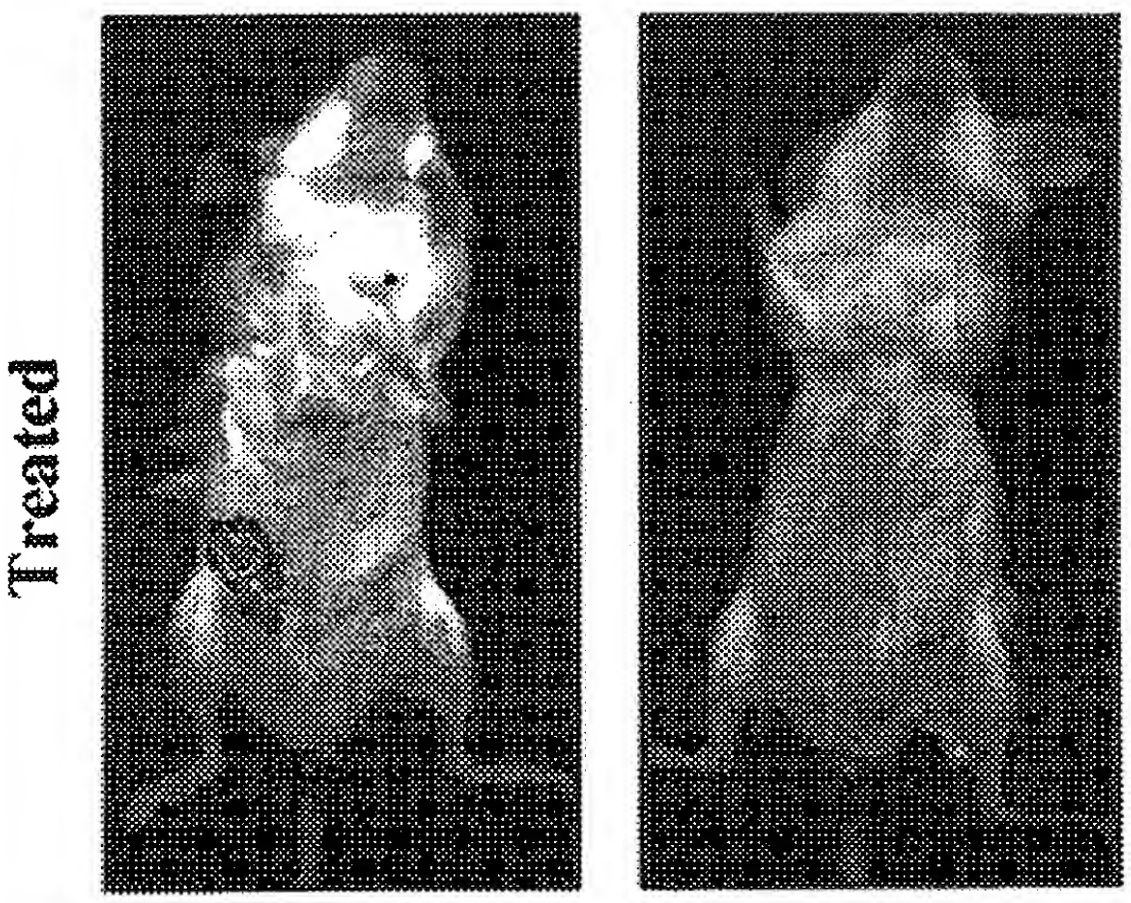


FIG. 11D FIG. 11E

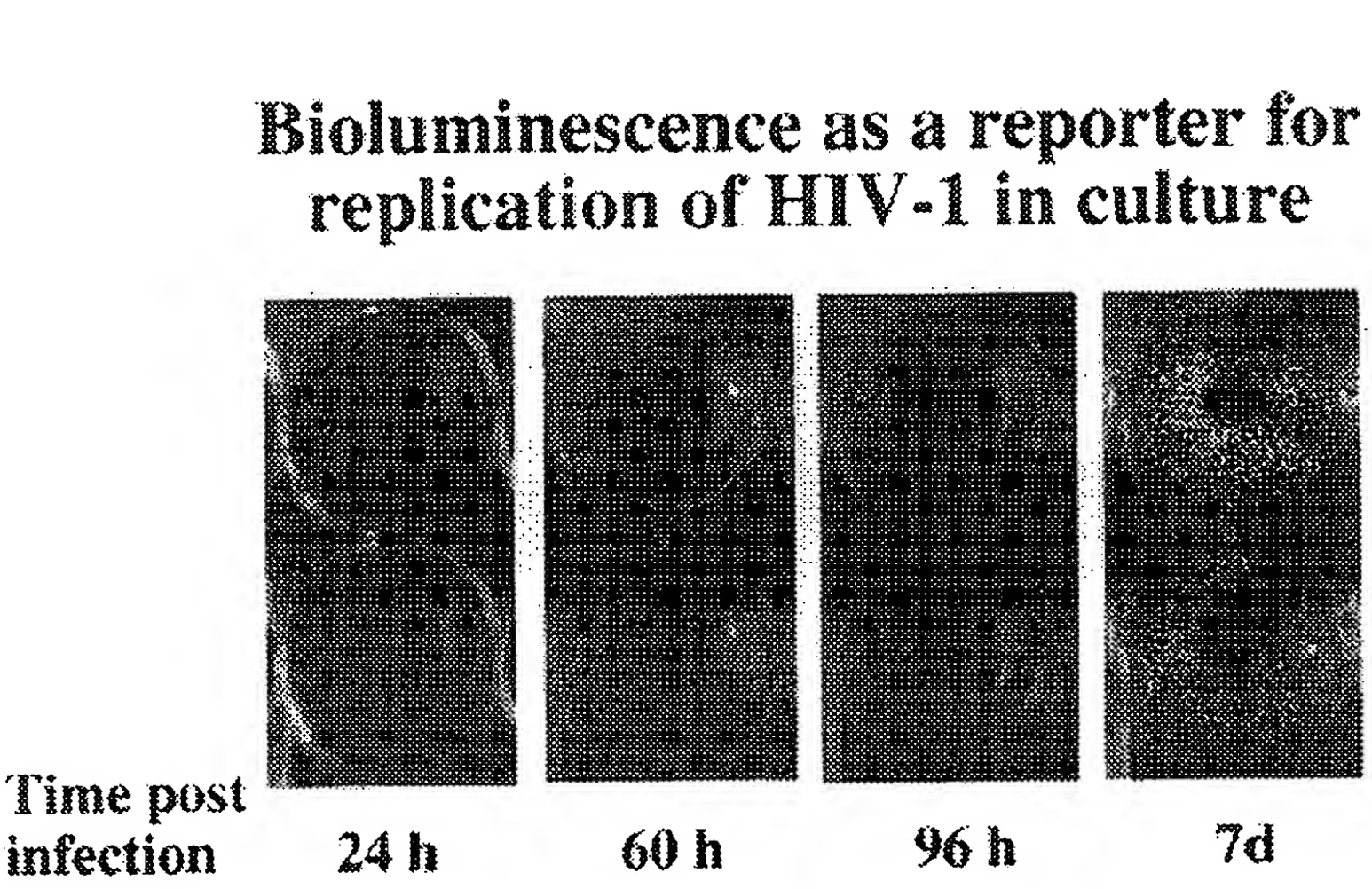

Bioluminescence as a reporter for replication of HIV-1 in culture
Time post infection
24 h
60 h
96 h
7d

FIG. 12

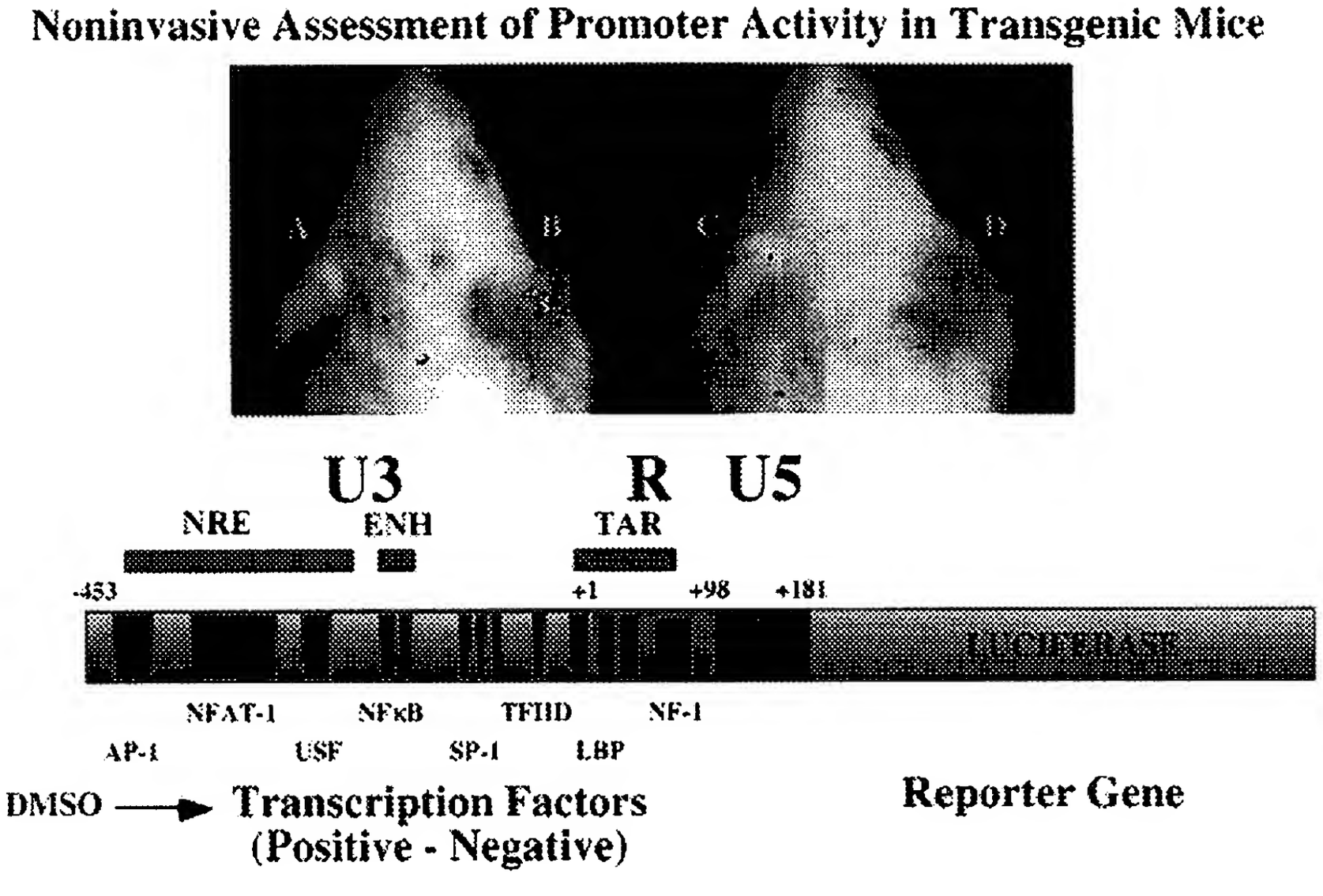

Noninvasive Assessment of Promoter Activity in Transgenic Mice
A
B
C
D
U3
R
U5
NRE
ENH
TAR
-453
+1
+98
+181
LUCIFERASE
NFAT-1
NFκB
TFIID
NF-1
AP-1
USF
SP-1
LBP
DMSO
Transcription Factors
(Positive - Negative)
Reporter Gene

FIG. 13

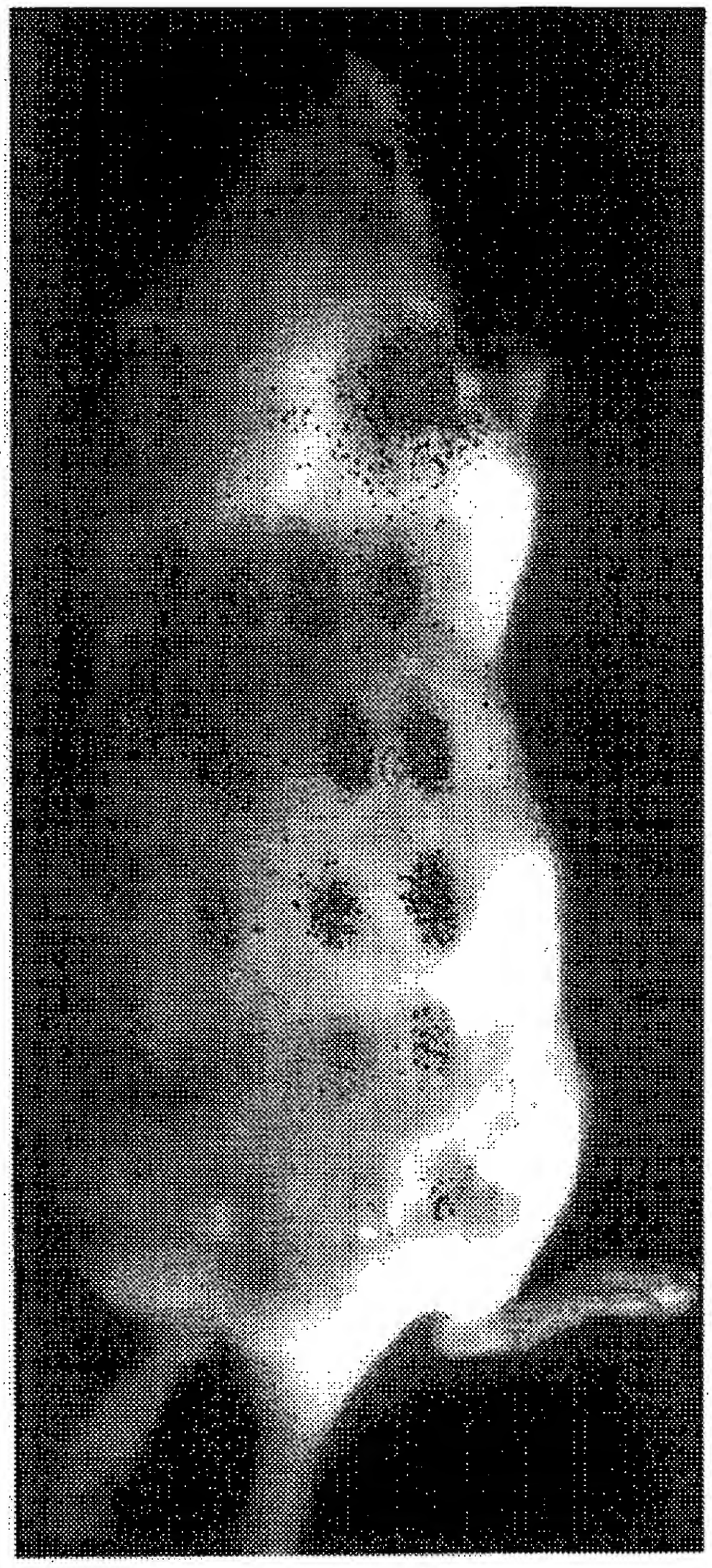


FIG. 14

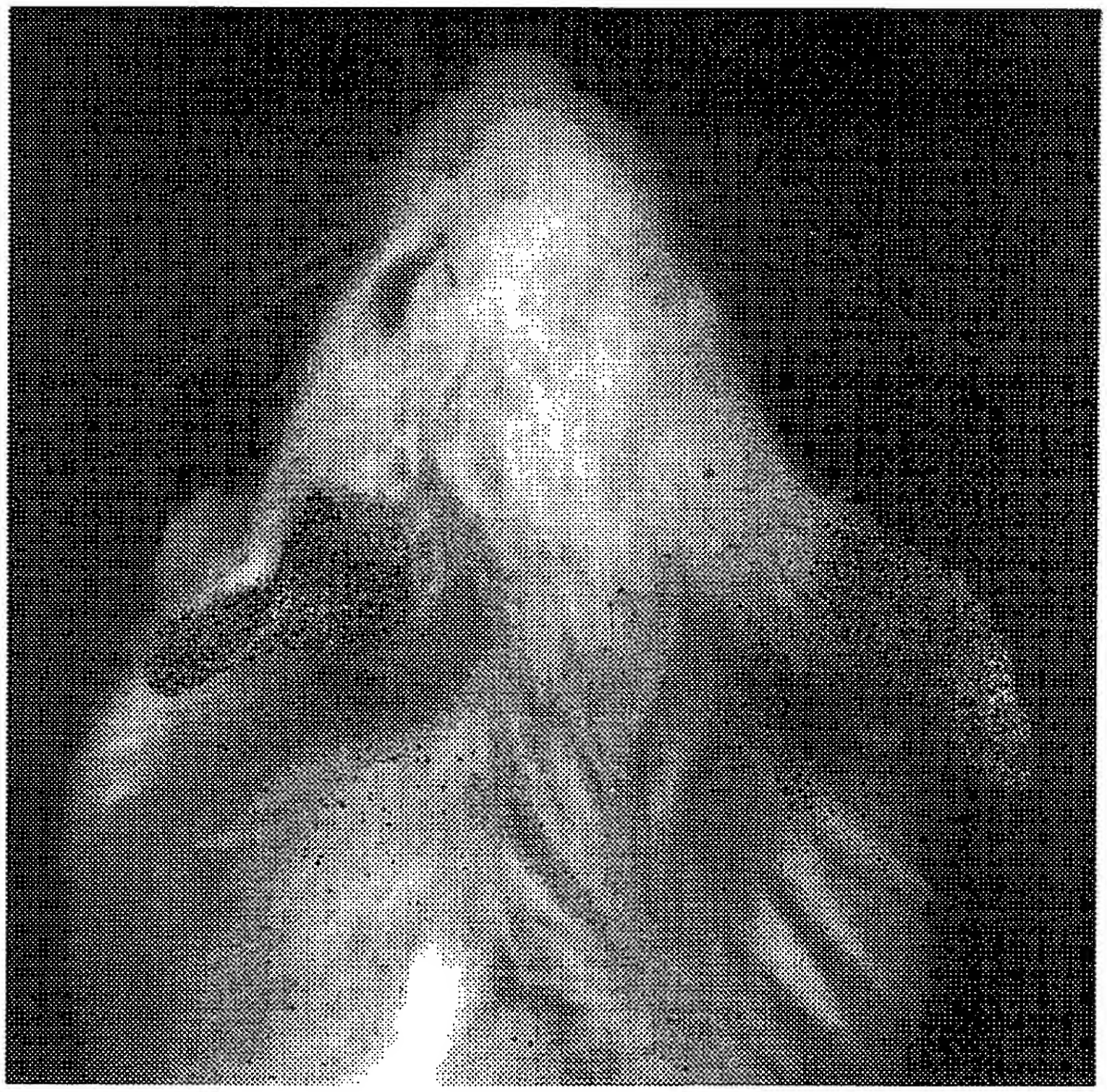


FIG. 15

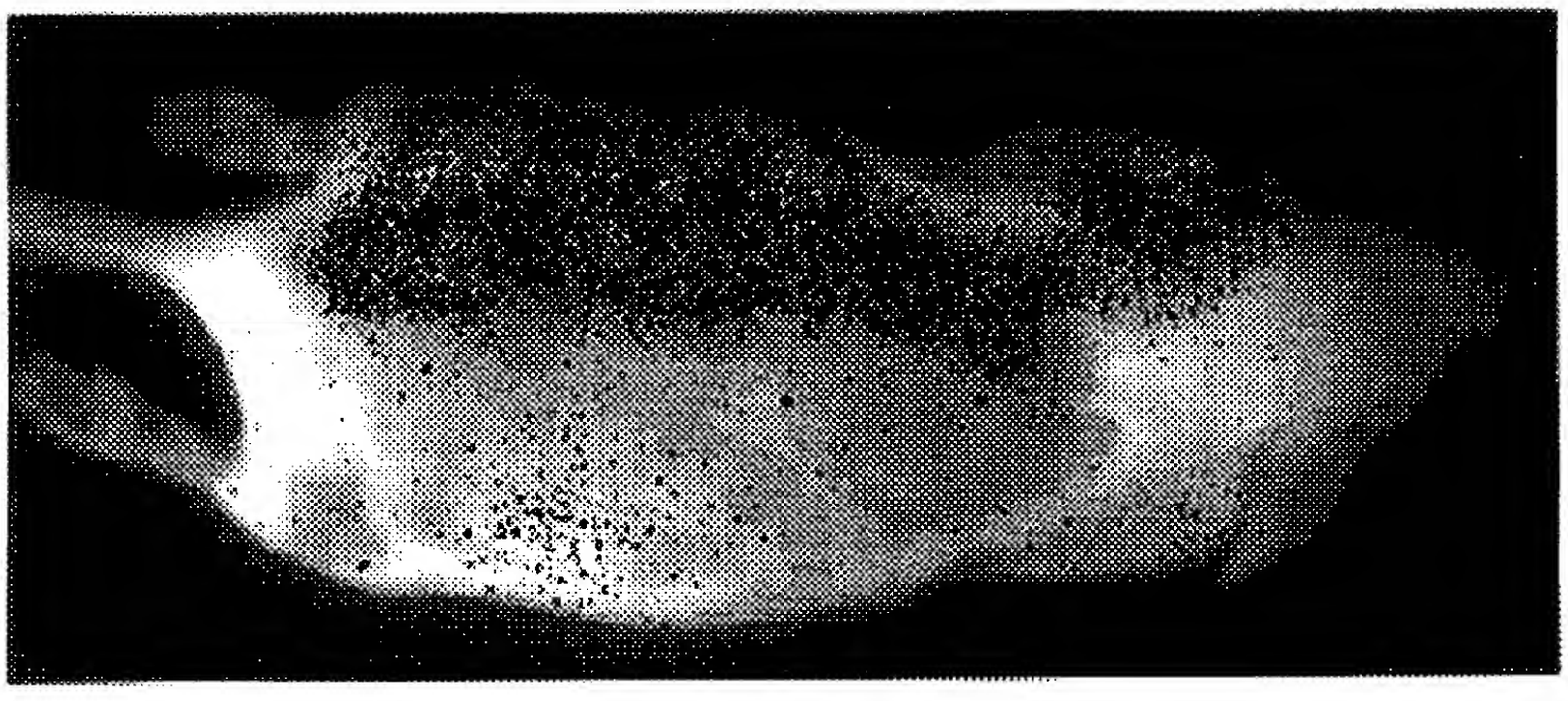


FIG. 16

FIG. 17

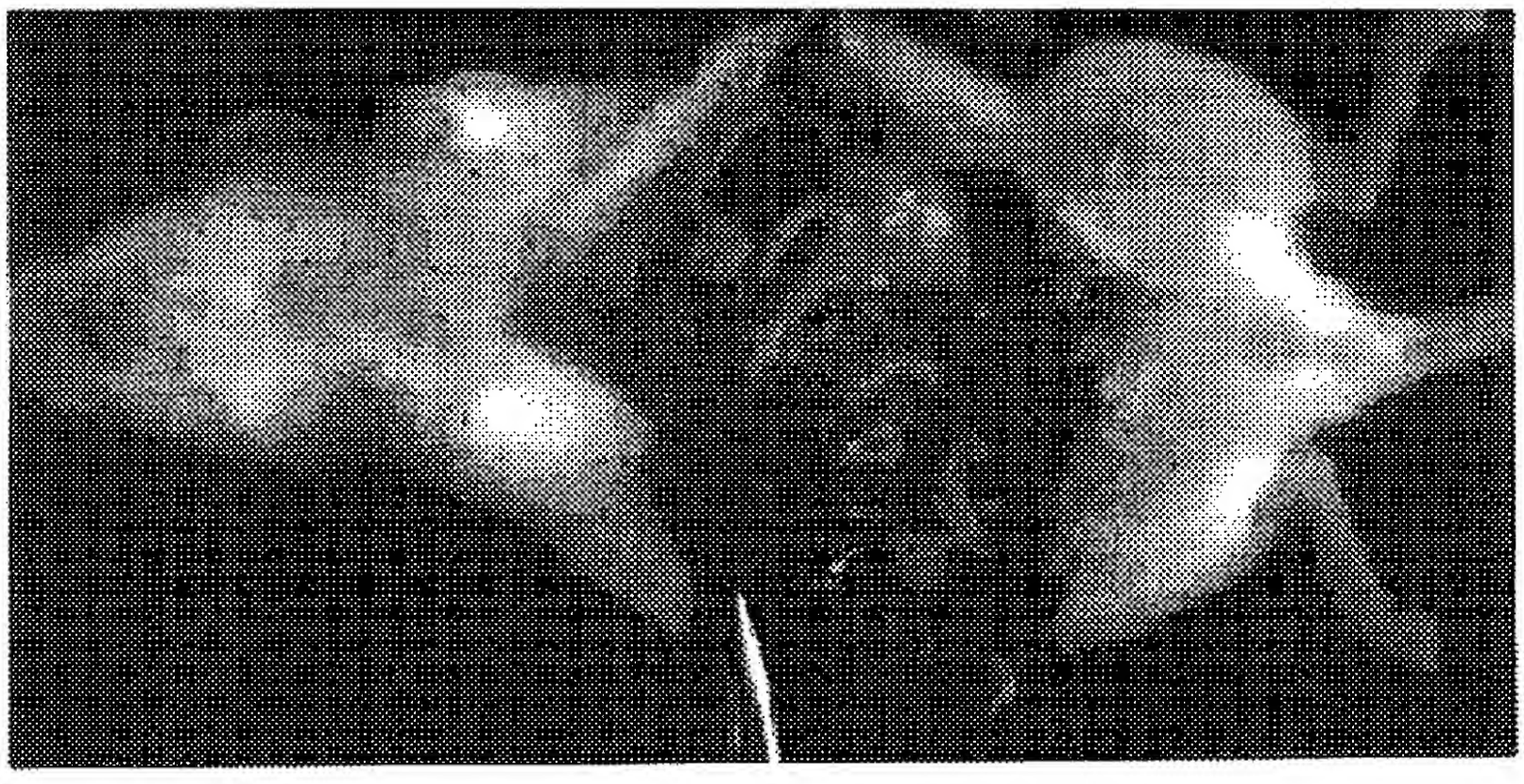


FIG. 18

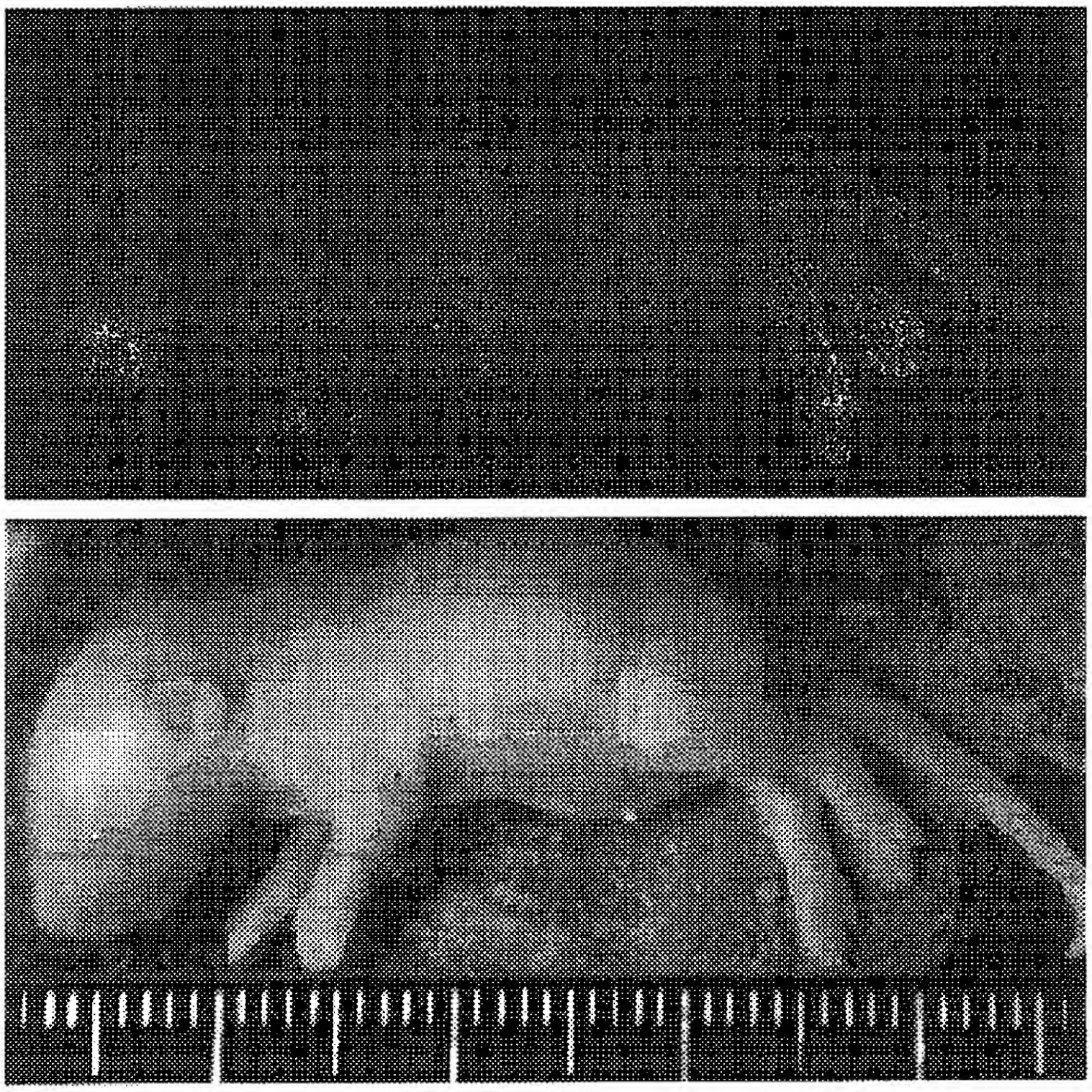


FIG. 19

NON-INVASIVE LOCALIZATION OF A LIGHT-EMITTING CONJUGATE IN A MAMMAL

This application is a divisional of co-owned U.S. patent application Ser. No. 08/602,396, filed Feb. 16, 1996, now abandoned, which is continuation-in-part of co-owned U.S. patent application Ser. No. 08/270,631, filed Jul. 1, 1994 (now U.S. Patent No. 5,650,135).

This work was supported in part by grants from the Office of Naval Research (N-00014-91-C-0170), the National Institutes of Health (PHS M01-RR-00070-30/1 and RR-00081) and the United States Public Health Service. Accordingly, the United States Government has certain rights in this invention.

I. FIELD OF THE INVENTION

The present invention relates to noninvasive methods and compositions for detecting, localizing and tracking light-emitting entities and biological events in a mammalian subject.

II. BACKGROUND OF THE INVENTION

The ability to monitor the progression of infectious diseases is limited by the current ex vivo methods of detecting and quantifying infectious agents in tissues. The replication of an infectious agent in a host often involves primary, secondary and tertiary sites of replication. The sites of replication and the course that an infectious agent follows through these sites is determined by the route of inoculation, factors encoded by the host as well as determinants of the infecting agent.

Experience may offer, in some cases, an estimate of probable sites of replication and the progress of an infection. It is more often the case, however, that the sites of infection, and the pace of the disease are either not known or can only roughly be estimated. Moreover, the progression of an infectious disease, even in inbred strains of mice, is often individualized, and serial, ex vivo analyses of many infected animals need to be conducted to determine, on the average, what course a disease will follow in an experimentally infected host.

Accordingly, it would be desirable to have a means of tracking the progression of infection in an animal model. Ideally, the tracking could be done non-invasively, such that a single animal could be evaluated as often as necessary without detrimental effects. Methods and compositions of the present invention provide a non-invasive approach to detect, localize and track a pathogen, as well as other entities, in a living host, such as a mammal.

III. SUMMARY OF THE INVENTION

In one embodiment, the invention includes a noninvasive method for detecting the localization of a biocompatible entity in a mammalian subject. The entity can be a molecule, macromolecule, cell, microorganism (including a pathogen), a particle, or the like.

The method includes administering to the subject a conjugate of the entity and a light-generating moiety. Light-generating moieties are typically molecules or macromolecules that give off light. They may generate light as a result of radiation absorption (e.g., fluorescent or phosphorescent molecules), or as a result of a chemical reaction (e.g., bioluminescent proteins). Exemplary light-generating moieties are bioluminescent proteins, such as luciferase and aequorin, and colored or fluorescent proteins, such as yellow fluorescent protein and ferredoxin IV.

The moiety may be conjugated to the entity by a variety of techniques, including incorporation during synthesis of the entity (e.g., chemical or genetic, such a fusion protein of an antibody fragment and a light-generating protein), chemical coupling post-synthesis, non-covalent association (e.g., encapsulation by liposomes), in-situ synthesis in the entity (e.g., expression of a heterologous bioluminescent protein in a transformed cell), or in situ activatable promoter-controlled expression of a bioluminescent protein in cells of a transgenic animal stimulated by a promoter inducer (e.g., interferon-activated promoter stimulated by infection with a virus).

After a period of time in which the conjugate can localize in the subject, the subject is immobilized within the detection field of a photodetector device for a period of time effective to measure a sufficient amount of photon emission (with the photodetector device) to construct an image. An exemplary photodetector device is an intensified charge-coupled device (ICCD) camera coupled to an image processor. If the image can be constructed in a time short relative to the time scale at which an "unimmobilized" subject moves, the subject is inherently "immobilized" during imaging and no special immobilization precautions are required. An image from the photon emission data is then constructed.

The method described above can be used to track the localization of the entity in the subject over time, by repeating the imaging steps at selected intervals and constructing images corresponding to each of those intervals.

The method described above can be used in a number of specific applications, by attaching, conjugating or incorporating targeting moieties onto the entity. The targeting moiety may be an inherent property of the entity (e.g., antibody or antibody fragment), or it may be conjugated to, attached to, or incorporated in the entity (e.g., liposomes containing antibodies). Examples of targeting moieties include antibodies, antibody fragments, enzyme inhibitors, receptor-binding molecules, various toxins and the like. Targets of the targeting moiety may include sites of inflammation, infection, thrombotic plaques and tumor cells. Markers distinguishing these targets, suitable for recognition by targeting moieties, are well known.

Further, the method may be used to detect and localize sites of infection by a pathogen in an animal model, using the pathogen (e.g., Salmonella) conjugated to a light-generating moiety as the entity.

In a related embodiment, the invention includes a noninvasive method for detecting the level of a biocompatible entity in a mammalian subject over time. The method is similar to methods described above, but is designed to detect changes in the level of the entity in the subject over time, without necessarily localizing the entity in the form of an image. This method is particularly useful for monitoring the effects of a therapeutic substance, such an antibiotic, on the levels of an entity, such as a light-emitting bacterium, over time.

In another embodiment, the invention includes a noninvasive method for detecting the integration of a transgene in a mammalian subject. The method includes administering to the subject, a vector construct effective to integrate a transgene into mammalian cells. Such constructs are well known in the art. In addition to the elements necessary to integrate effectively, the construct contains a transgene (e.g., a therapeutic gene), and a gene encoding a light-generating protein

under the control of a selected activatable promoter. After a period of time in which the construct can achieve integration, the promoter is activated. For example, if an interferon inducible promoter is used, a poly-inosine and -cytosine duplex (poly-IC) can be locally administered (e.g., footpad injection) to stimulate interferon production. The HIV LTR could similarly be used and induced, for example, with dimethylsulfoxide (DMSO). The subject is then placed within the detection field of a photodetector device, such as an individual wearing light-intensifying "night vision" goggles, and the level of photon emission is measured, or evaluated. If the level is above background (i.e., if light can be preferentially detected in the "activated" region), the subject is scored as having integrated the transgene.

In a related embodiment, the invention includes a noninvasive method for detecting the localization of a promoter-induction event in an animal made transgenic or chimeric for a construct including a gene encoding a light-generating protein under the control of an inducible promoter. Promoter induction events include the administration of a substance which directly activates the promoter, the administration of a substance which stimulates production of an endogenous promoter activator (e.g., stimulation of interferon production by RNA virus infection), the imposition of conditions resulting in the production of an endogenous promoter activator (e.g., heat shock or stress), and the like. The event is triggered, and the animal is imaged as described above.

In yet another embodiment, the invention includes pathogens, such as Salmonella, transformed with a gene expressing a light-generating protein, such as luciferase.

In another aspect, the invention includes a method of identifying therapeutic compounds effective to inhibit spread of infection by a pathogen. The method includes administering a conjugate of the pathogen and a light-generating moiety to control and experimental animals, treating the experimental animals with a putative therapeutic compound, localizing the light-emitting pathogen in both control and experimental animals by the methods described above, and identifying the compound as therapeutic if the compound is effective to significantly inhibit the spread or replication of the pathogen in the experimental animals relative to control animals. The conjugates include a fluorescently-labeled antibodies, fluorescently-labeled particles, fluorescently-labeled small molecules, and the like.

In still another aspect, the invention includes a method of localizing entities conjugated to light-generating moieties through media of varying opacity. The method includes the use of photodetector device to detect photons transmitted through the medium, integrate the photons over time, and generate an image based on the integrated signal.

In yet another embodiment, the invention includes a method of measuring the concentration of selected substances, such as dissolved oxygen or calcium, at specific sites in an organism. The method includes entities, such as cells, containing a concentration sensor—a light-generating molecule whose ability to generate light is dependent on the concentration of the selected substance. The entity containing the light-generating molecule is administered such that it adopts a substantially uniform distribution in the animal or in a specific tissue or organ system (e.g., spleen). The organism is imaged, and the intensity and localization of light emission is correlated to the concentration and location of the selected substance. Alternatively, the entity contains a second marker, such as a molecule capable of generating light at a wavelength other than the concentration sensor. The second marker is used to normalize for any non-uniformities in the distribution of the entity in the host, and thus permit a more accurate determination of the concentration of the selected substance.

In another aspect, the invention includes a method of identifying therapeutic compounds effective to inhibit the growth and/or the metastatic spread of a tumor. The method includes (i) administering tumor cells labeled with or containing light-generating moieties to groups of experimental and control animals, (ii) treating the experimental group with a selected compound, (iii) localizing the tumor cells in animals from both groups by imaging photon emission from the light-generating molecules associated with the tumor cells with a photodetector device, and (iv) identifying a compound as therapeutic if the compound is able to significantly inhibit the growth and/or metastatic spread of the tumor in the experimental group relative to the control group.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color.

FIGS. 1A, 1B and 1C show a map of the lux pCGLS1 plasmid used to transform Salmonella strains SL1344, BJ66 and LB5000 to generate strains SL1344lux, BJ66lux and LB5000lux. FIG. 1A depicts a restriction enzyme map of the lux operon, which is inserted into the BamHI site of the polylinker depicted in FIG. 1B. A sequence included in the multiple cloning site (MCS) is provided in FIG. 1B as SEQ ID NO:1, with the Bam HI site indicated in bold type. A graphical representation of a pUC1 vector without insert is shown in FIG. 1C.

FIGS. 2A, 2B, 2C, 2D and 2E depict the adherence and invasion of Salmonella strains SL1344lux and BJ66lux on macrophages and HEp-2 cells.

FIG. 2A depicts luminescent bacterial cells localized in wells of an assay dish. The pseudo-color image, obtained by integrating photons over one minute, is superimposed over a gray scale image of the assay dish, producing the "composite image" shown.

FIG. 2B depicts the relative light intensity of wells that were not treated with gentamicin.

FIG. 2C depicts the number of colony forming units (CFU) per ml isolated from the same wells as were imaged in FIG. 2B.

FIG. 2D depicts the relative light intensity of wells that were treated with gentamicin.

FIG. 2E depicts the number of colony forming units (CFU) per ml isolated from the same wells as were imaged in FIG. 2D.

FIG. 3A depicts a composite image of four glass capillary tubes containing dilutions of LB5000lux bacterial suspensions. Luminescence was determined by integrating over 30 seconds. Air pockets are present in each tube on both sides of the suspension.

FIG. 3B depicts the distribution of bioluminescence following intraperitoneal inoculation of wild-type SL1344lux into mice.

FIG. 4 depicts the effect of human blood on the light emission from bioluminescent Salmonella.

FIG. 5 depicts a schematic diagram of a vial used to test the transmission of light generated by LBSOOOlux through animal tissue.

FIG. 6A depicts composite images of Balb/c mice orally inoculated with low virulence LB5000lux Salmonella, and imaged at the times indicated. The luminescence signal was integrated over 5 minutes.

FIG. 6B depicts composite images of Balb/c mice orally inoculated withnon-invasive BJ66lux Salmonella, and imaged at the times indicated. The luminescence signal was integrated over 5 minutes.

FIG. 6C depicts composite images of Balb/c mice orally inoculated with virulent SL1344lux Salmonella, and imaged at the times indicated. The luminescence signal was integrated over 5 minutes.

FIG. 7 depicts a composite image showing the distribution of Salmonella in mice 32 hours following intraperitoneal (i.p.) injections with either virulent SL1344lux (left two animals) or low virulence LB5000lux (right two animals) strains of the bacterium.

FIG. 8A depicts the distribution of virulent Salmonella in mice resistant to systemic Salmonella infections (129×Balb/c, $It^{r/s}$) on day one (1).

FIG. 8B depicts the distribution of virulent Salmonella in mice resistant to systemic Salmonella infections (129×Balb/c, $It^{r/s}$) on day eight (8).

FIGS. 9A, 9B, and 9C depict the distribution of mutant Salmonella with reduced virulence (BJ66lux) seven days following oral inoculation.

FIG. 9A depicts external, non-invasive imaging of the luminescence.

FIG. 9B depicts the same animal imaged following laparotomy. Labeled organs are cecum (C), liver (L), small intestine (I), and spleen (Sp).

FIG. 9C depicts a post-laparotomy image generated following injection of air into the lumen of the intestine both anterior and posterior to the cecum.

FIGS. 10A, 10B and 10C depict the distribution of Salmonella SL1344lux in susceptible Balb/c mice following intraperitoneal inoculation with SL1344lux.

FIG. 10A depicts an image prior to the opening of the peritoneal cavity.

FIG. 10B depicts an image after the opening of the peritoneal cavity.

FIG. 10C depicts an image after the cecum was pulled to the left side.

FIGS. 11A, 11B, 11C, 11D, and 11E depict the effects of ciprofloxacin treatment on bioluminescence from SL1344lux Salmonella in orally-inoculated mice.

FIG. 11A shows a graph of the relative bioluminescence intensity, measured from the abdominal area, as a function of time after initiation of treatment, for treated and untreated animals.

FIGS. 11B and 11D depict composite images of mice 8 days after oral inoculation with SL1344lux Salmonella, before treatment with ciprofloxacin.

FIGS. 11C and 11E depict composite images of the same mice 5.5 hours either following treatment (FIG. 11E) or control (no treatment: FIG. 11C).

FIG. 12 depicts bioluminescence as a reporter for replication of HIV-1 in culture. The gray scale image of the plates at 24 h, 60 h, 96 h, and 7 d, as indicated, is shown.

FIG. 13 depicts an assessment of the promoter activity in tissues of transgenic mice containing a construct composed of the regulatory portion of the HIV LTR (U3 region) upstream of the coding sequence of the firefly luciferase gene. NRE, negative response element; ENH, enhancer region, TAR-transactivation responsive element.

FIG. 14 depicts topical delivery of substrate to dermal cells in transgenic mice containing a construct composed of the regulatory portion of the HIV LTR upstream of the coding sequence of the firefly luciferase gene.

FIG. 15 depicts bioluminescence from induced ears as result of topical luciferin delivery in transgenic mice containing a construct composed of the regulatory portion of the HIV LTR upstream of the coding sequence of the firefly luciferase gene.

FIG. 16 depicts unilateral induction of luciferase expression in transgenic mice; the left half of the shaved dorsal surface of the mice and the left ear were treated with DMSO to activate expression of the HIV-1 LTR; luciferin was applied topically over the entire surface of the back and both ears.

FIG. 17 depicts the detection of bioluminescence from internal tissues in transgenic mice.

FIG. 18 depicts imaging of the abdomen of animals following laprotomy demonstrating signals to localize the origin of internal bioluminescence.

FIG. 19 depicts expression of the differential expression of HIV-LTR in neonatal transgenic mice.

V. DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art of the present invention.

Opaque medium is used herein to refer to a medium that is "traditionally" opaque, not necessarily absolutely opaque. Accordingly, an opaque medium is defined as a medium that is commonly considered to be neither transparent nor translucent, and includes items such as a wood board, and flesh and skin of a mammal.

Luciferase, unless stated otherwise, includes prokaryotic and eukaryotic luciferases, as well as variants possessing varied or altered optical properties, such as luciferases that luminesce at wavelengths in the red range.

Biocompatible entity is an entity that can be administered to a mammal. This includes pathogens which may be deleterious to the mammal. In reference to an animal whose cells contain a transgene expressing a light-generating protein, biocompatible entity refers to the transgene-containing cells comprising the mammal.

Light-generating is defined as capable of generating light through a chemical reaction or through the absorption of radiation.

Light is defined herein, unless stated otherwise, as electromagnetic radiation having a wavelength of between about 300 nm and about 1100 nm.

Spread of infection typically refers to the spreading and colonization by a pathogen of host sites other than the initial infection site. The term can also include, however, growth in size and/or number of the pathogen at the initial infection site.

lux—prokaryotic genes associated with luciferase and photon emission.

luc—eukaryotic genes associated with luciferase and photon emission.

Promoter induction event refers to an event that results in the direct or indirect induction of a selected inducible promoter.

Heterologous gene refers to a gene which has been transfected into a host organism. Typically, a heterologous

gene refers to a gene that is not originally derived from the transfected or transformed cells, genomic DNA.

Transgene refers to a heterologous gene which has been introduced, transiently or permanently, into the germ line or somatic cells of an organism.

B. General Overview Of The Invention

The present invention includes methods and compositions relating to non-invasive imaging and/or detecting of light-emitting conjugates in mammalian subjects. The conjugates contain a biocompatible entity and a light-generating moiety. Biocompatible entities include, but are not limited to, small molecules such as cyclic organic molecules; macromolecules such as proteins; microorganisms such as viruses, bacteria, yeast and fungi; eukaryotic cells; all types of pathogens and pathogenic substances; and particles such as beads and liposomes. In another aspect, biocompatible entities may be all or some of the cells that constitute the mammalian subject being imaged.

Light-emitting capability is conferred on the entities by the conjugation of a light-generating moiety. Such moieties include fluorescent molecules, fluorescent proteins, enzymatic reactions giving off photons and luminescent substances, such as bioluminescent proteins. The conjugation may involve a chemical coupling step, genetic engineering of a fusion protein, or the transformation of a cell, microorganism or animal to express a bioluminescent protein. For example, in the case where the entities are the cells constituting the mammalian subject being imaged, the light-generating moiety may be a bioluminescent or fluorescent protein "conjugated" to the cells through localized, promoter-controlled expression from a vector construct introduced into the cells by having made a transgenic or chimeric animal.

Light-emitting conjugates are typically administered to a subject by any of a variety of methods, allowed to localize within the subject, and imaged. Since the imaging, or measuring photon emission from the subject, may last up to tens of minutes, the subject is usually, but not always, immobilized during the imaging process.

Imaging of the light-emitting entities involves the use of a photodetector capable of detecting extremely low levels of light—typically single photon events—and integrating photon emission until an image can be constructed. Examples of such sensitive photodetectors include devices that intensify the single photon events before the events are detected by a camera, and cameras (cooled, for example, with liquid nitrogen) that are capable of detecting single photons over the background noise inherent in a detection system.

Once a photon emission image is generated, it is typically superimposed on a "normal" reflected light image of the subject to provide a frame of reference for the source of the emitted photons (i.e., localize the light-emitting conjugates with respect to the subject). Such a "composite" image is then analyzed to determine the location and/or amount of a target in the subject.

The steps and embodiments outlined above are presented in greater detail, below.

C. Light-Emitting Entities

1. Light-Generating Moieties.

The light-generating moieties (LGMs), molecules or constructs useful in the practice of the present invention may take any of a variety of forms, depending on the application. They share the characteristic that they are luminescent, that is, that they emit electromagnetic radiation in ultraviolet (UV), visible and/or infra-red (IR) from atoms or molecules as a result of the transition of an electronically excited state to a lower energy state, usually the ground state.

Examples of light-generating moieties include photoluminescent molecules, such as fluorescent molecules, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds.

Two characteristics of LGMs that bear considerable relevance to the present invention are their size and their spectral properties. Both are discussed in the context of specific types of light-generating moieties described below, following a general discussion of spectral properties.

Spectral Properties. An important aspect of the present invention is the selection of light-generating moieties that produce light capable of penetrating animal tissue such that it can be detected externally in a non-invasive manner. The ability of light to pass through a medium such as animal tissue (composed mostly of water) is determined primarily by the light's intensity and wavelength.

The more intense the light produced in a unit volume, the easier the light will be to detect. The intensity of light produced in a unit volume depends on the spectral characteristics of individual LGMs, discussed below, and on the concentration of those moieties in the unit volume. Accordingly, conjugation schemes that place a high concentration of LGMs in or on an entity (such as high-efficiency loading of a liposome or high-level expression of a bioluminescent protein in a cell) typically produce brighter light-emitting conjugates (LECs), which are easier to detect through deeper layers of tissue, than schemes which conjugate, for example, only a single LGM onto each entity.

A second factor governing the detectability of an LGM through a layer of tissue is the wavelength of the emitted light. Water may be used to approximate the absorption characteristics of animal tissue, since most tissues are composed primarily of water. It is well known that water transmits longer-wavelength light (in the red range) more readily than it does shorter wavelength light.

Accordingly, LGMs which emit light in the range of yellow to red (550–1100 nm) are typically preferable to LGMs which emit at shorter wavelengths. Several of the LGMs discussed below emit in this range. However, it will be noted, based on experiments performed in support of the present invention and presented below, that excellent results can be achieved in practicing the present invention with LGMs that emit in the range of 486 nm, despite the fact that this is not an optimal emission wavelength. These results are possible, in part, due to the relatively high concentration of LGMs (luciferase molecules) present in the LECs (transformed Salmonella cells) used in these experiments, and to the use of a sensitive detector. It will be understood that through the use of LGMs with a more optimal emission wavelength, similar detection results can be obtained with LGEs having lower concentrations of the LGMs.

Fluorescence-based Moieties. Fluorescence is the luminescence of a substance from a single electronically excited state, which is of very short duration after removal of the source of radiation. The wavelength of the emitted fluorescence light is longer than that of the exciting illumination (Stokes' Law), because part of the exciting light is converted into heat by the fluorescent molecule.

Because fluorescent molecules require input of light in order to luminesce, their use in the present invention may be more complicated than the use of bioluminescent molecules. Precautions are typically taken to shield the excitatory light so as not to contaminate the fluorescence photon signal being detected from the subject. Obvious precautions

include the placement of an excitation filter, such that employed in fluorescence microscope, at the radiation source. An appropriately-selected excitation filter blocks the majority of photons having a wavelength similar to that of the photons emitted by the fluorescent moiety. Similarly a barrier filter is employed at the detector to screen out most of the photons having wavelengths other than that of the fluorescence photons. Filters such as those described above can be obtained from a variety of commercial sources, including Omega Optical, Inc. (Brattleboro, Vt.).

Alternatively, a laser producing high intensity light near the appropriate excitation wavelength, but not near the fluorescence emission wavelength, can be used to excite the fluorescent moieties. An x-y translation mechanism may be employed so that the laser can scan the subject, for example, as in a confocal microscope.

As an additional precaution, the radiation source can be placed behind the subject and shielded, such that the only radiation photons reaching the site of the detector are those that pass all the way through the subject. Furthermore, detectors may be selected that have a reduced sensitivity to wavelengths of light used to excite the fluorescent moiety.

Through judicious application of the precautions above, the detection of fluorescent LGMs according to methods of the present invention is possible.

Fluorescent moieties include small fluorescent molecules, such as fluorescein, as well as fluorescent proteins, such as green fluorescent protein (Chalfie, et al., 1994, *Science* 263:802–805., Morin and Hastings, 1971, *J. Cell. Physiol.* 77:313) and lumazine and yellow fluorescent proteins (O'Kane, et al., 1991, *PNAS* 88:1100–1104, Daubner, et al., 1987, *PNAS* 84:8912–8916). In addition, certain colored proteins such as ferredoxin IV (Grabau, et al., 1991, *J Biol Chem.* 266:3294–3299), whose fluorescence characteristics have not been evaluated, may be fluorescent and thus applicable for use with the present invention. Ferredoxin IV is a particularly promising candidate, as it has a reddish color, indicating that it may fluoresce or reflect at a relatively long wavelength and produce light that is effective at penetrating tissue. Furthermore, the molecule is small for a protein (95 amino acids), and can thus be conjugated to entities with a minimal impact on their function.

An advantage of small fluorescent molecules is that they are less likely to interfere with the bioactivity of the entity to which they are attached than a would a larger light-generating moiety. In addition, commercially-available fluorescent molecules can be obtained with a variety of excitation and emission spectra that are suitable for use with the present invention. For example, Molecular Probes (Eugene, Oreg.) sells a number of fluorophores, including Lucifer Yellow (abs. at 428 nm, and emits at 535 nm) and Nile Red (abs. at 551 nm and emits at 636 nm). Further, the molecules can be obtained derivatized with a variety of groups for use with various conjugation schemes (e.g., from Molecular Probes).

Bioluminescence-Based Moieties. The subjects of chemiluminescence (luminescence as a result of a chemical reaction) and bioluminescence (visible luminescence from living organisms) have, in many aspects, been thoroughly studied (e.g., Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). A brief summary of salient features follows.

Bioluminescent molecules are distinguished from fluorescent molecules in that they do not require the input of radiative energy to emit light. Rather, bioluminescent molecules utilize chemical energy, such as ATP, to produce light. An advantage of bioluminescent moieties, as opposed to fluorescent moieties, is that there is virtually no background in the signal. The only light detected is light that is produced by the exogenous bioluminescent moiety. In contrast, the light used to excite a fluorescent molecule often results in the fluorescence of substances other than the intended target. This is particularly true when the "background" is as complex as the internal environment of a living animal.

Several types of bioluminescent molecules are known. They include the luciferase family (e.g., Wood, et al., 1989, *Science* 244:700–702) and the aequorin family (e.g., Prasher, et al., *Biochem.* 26:1326–1332). Members of the luciferase family have been identified in a variety of prokaryotic and eukaryotic organisms. Luciferase and other enzymes involved in the prokaryotic luminescent (lux) systems, as well as the corresponding lux genes, have been isolated from marine bacteria in the Vibrio and Photobacterium genera and from terrestrial bacteria in the Xenorhabdus genus.

An exemplary eukaryotic organism containing a luciferase system (luc) is the North American firefly *Photinus pyralis*. Firefly luciferase has been extensively studied, and is widely used in ATP assays. cDNAs encoding luciferases from *Pyrophorus plagiophthalamus*, another species of click beetle, have been cloned and expressed (Wood, et al., 1989, *Science* 244:700–702). This beetle is unusual in that different members of the species emit bioluminescence of different colors. Four classes of clones, having 95–99% homology with each other, were isolated. They emit light at 546 nm (green), 560 nm (yellow-green), 578 nm (yellow) and 593 nm (orange). The last class (593 nm) may be particularly advantageous for use as a light-generating moiety with the present invention, because the emitted light has a wavelength that penetrates tissues more easily than shorter wavelength light.

Luciferases, as well as aequorin-like molecules, require a source of energy, such as ATP, NAD(P)H, and the like, and a substrate, such as luciferin or coelentrizine and oxygen.

The substrate luciferin must be supplied to the luciferase enzyme in order for it to luminesce. In those cases where a luciferase enzyme is introduced as an expression product of a vector containing cDNA encoding a lux luciferase, a convenient method for providing luciferin is to express not only the luciferase but also the biosynthetic enzymes for the synthesis of luciferin. In cells transformed with such a construct, oxygen is the only extrinsic requirement for bioluminescence. Such an approach, detailed in Example 1, is employed to generate lux-transformed Salmonella, which are used in experiments performed in support of the present invention and detailed herein.

The plasmid construct, encoding the lux operon obtained from the soil bacterium *Xenorhabdus luminescens* (Frackman, et al., 1990, *J. Bact.* 172:5767–5773), confers on transformed *E coli* the ability to emit photons through the expression of the two subunits of the heterodimeric luciferase and three accessory proteins (Frackman, et al., 1990). Optimal bioluminescence for *E. Coli* expressing the lux genes of *X. luminescens* is observed at 37° C. (Szittner and Meighen, 1990, *J. Biol. Chem.* 265:16581–16587, Xi, et al., 1991, *J. Bact.* 173:1399–1405) in contrast to the low temperature optima of luciferases from eukaryotic and other prokaryotic luminescent organisms (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and

VCH Verlagsgesellschaft mbH)). The luciferase from *X. luminescens*, therefore, is well-suited for use as a marker for studies in animals.

Luciferase vector constructs such as the one described above and in Example 1, can be adapted for use in transforming a variety of host cells, including most bacteria, and many eukaryotic cells (luc constructs). In addition, certain viruses, such as herpes virus and vaccinia virus, can be genetically-engineered to express luciferase. For example, Kovacs Sz. and Mettenlieter, 1991, *J. Gen. Virol.* 72:2999–3008, teach the stable expression of the gene encoding firefly luciferase in a herpes virus. Brasier and Ron, 1992, *Meth. in Enzymol.* 216:386–396, teach the use of luciferase gene constructs in mammalian cells. Luciferase expression from mammalian cells in culture has been studied using CCD imaging both macroscopically (Israel and Honigman, 1991, Gene 104:139–145) and microscopically (Hooper, et al., 1990, *J. Biolum. and Chemilum.* 5:123–130).

2. Entities

The invention includes entities which have been modified or conjugated to include a light-generating moiety, construct or molecule, such as described above. Such conjugated or modified entities are referred to as light-emitting entities, light-emitting conjugates (LECs) or simply conjugates. The entities themselves may take the form of, for example, molecules, macromolecules, particles, microorganisms, or cells. The methods used to conjugate a light-generating moiety to an entity depend on the nature of the moiety and the entity. Exemplary conjugation methods are discussed in the context of the entities described below.

Small molecules. Small molecule entities which may be useful in the practice of the present invention include compounds which specifically interact with a pathogen or an endogenous ligand or receptor. Examples of such molecules include, but are not limited to, drugs or therapeutic compounds; toxins, such as those present in the venoms of poisonous organisms, including certain species of spiders, snakes, scorpions, dinoflagellates, marine snails and bacteria; growth factors, such as NGF, PDGF, TGF and TNF; cytokines; and bioactive peptides.

The small molecules are preferably conjugated to light-generating moieties that interfere only minimally, if at all, with the bioactivity of the small molecule, such as small fluorescent molecules (described above). Conjugations are typically chemical in nature, and can be performed by any of a variety of methods known to those skilled in the art.

The small molecule entity may be synthesized to contain a light-generating moiety, so that no formal conjugation procedure is necessary. Alternatively, the small molecule entity may be synthesized with a reactive group that can react with the light generating moiety, or vice versa.

Small molecules conjugated to light-generating moieties of the present invention may be used either in animal models of human conditions or diseases, or directly in human subjects to be treated. For example, a small molecule which binds with high affinity to receptor expressed on tumor cells may be used in an animal model to localize and obtain size estimates of tumors, and to monitor changes in tumor growth or metastasis following treatment with a putative therapeutic agent. Such molecules may also be used to monitor tumor characteristics, as described above, in cancer patients.

Macromolecules. Macromolecules, such as polymers and biopolymers, constitute another example of entities useful in practicing the present invention. Exemplary macromolecules include antibodies, antibody fragments, fusion proteins and certain vector constructs.

Antibodies or antibody fragments, purchased from commercial sources or made by methods known in the art (Harlow, et al., 1988, *Antibodies: A Laboratory Manual, Chapter* 10, pg. 402, Cold Spring Harbor Press), can be used to localize their antigen in a mammalian subject by conjugating the antibodies to a light-generating moiety, administering the conjugate to a subject by, for example, injection, allowing the conjugate to localize to the site of the antigen, and imaging the conjugate.

Antibodies and antibody fragments have several advantages for use as entities in the present invention. By their nature, they constitute their own targeting moieties. Further, their size makes them amenable to conjugation with several types of light-generating moieties, including small fluorescent molecules and fluorescent and bioluminescent proteins, yet allows them to diffuse rapidly relative to, for example, cells or liposomes.

The light-generating moieties can be conjugated directly to the antibodies or fragments, or indirectly by using, for example, a fluorescent secondary antibody. Direct conjugation can be accomplished by standard chemical coupling of, for example, a fluorophore to the antibody or antibody fragment, or through genetic engineering. Chimeras, or fusion proteins can be constructed which contain an antibody or antibody fragment coupled to a fluorescent or bioluminescent protein. For example, Casadei, et al., 1990, *PNAS* 87:2047–2051, describe a method of making a vector construct capable of expressing a fusion protein of aequorin and an antibody gene in mammalian cells.

Conjugates containing antibodies can be used in a number of applications of the present invention. For example, a labeled antibody directed against E-selectin, which is expressed at sites of inflammation, can be used to localize the inflammation and to monitor the effects of putative anti-inflammatory agents.

Vector constructs by themselves can also constitute macromolecular entities applicable to the present invention. For example, a eukaryotic expression vector can be constructed which contains a therapeutic gene and a gene encoding a light-generating molecule under the control of a selected promoter (i.e., a promoter which is expressed in the cells targeted by the therapeutic gene). Expression of the light-generating molecule, assayed using methods of the present invention, can be used to determine the location and level of expression of the therapeutic gene. This approach may be particularly useful in cases where the expression of the therapeutic gene has no immediate phenotype in the treated individual or animal model.

Viruses. Another entity useful for certain aspects of the invention are viruses. As many viruses are pathogens which infect mammalian hosts, the viruses may be conjugated to a light-generating moiety and used to study the initial site and spread of infection. In addition, viruses labeled with a light-generating moiety may be used to screen for drugs which inhibit the infection or the spread of infection.

A virus may be labeled indirectly, either with an antibody conjugated to a light-generating moiety, or by, for example, biotinylating virions (e.g., by the method of Dhawan, et al., 1991, *J. Immunol.* 147(1):102) and then exposing them to streptavidin linked to a detectable moiety, such as a fluorescent molecule.

Alternatively, virions may be labeled directly with a fluorophore like rhodamine, using, for example, the methods of Fan, et al., 1992, *J. Clin. Micro.* 30(4):905. The virus can also be genetically engineered to express a light-generating protein. The genomes of certain viruses, such as herpes and

vaccinia, are large enough to accommodate genes as large as the lux or luc genes used in experiments performed in support of the present invention.

Labeled virus can be used in animal models to localize and monitor the progression of infection, as well as to screen for drugs effective to inhibit the spread of infection. For example, while herpes virus infections are manifested as skin lesions, this virus can also cause herpes encephalitis. Such an infection can be localized and monitored using a virus labeled by any of the methods described above, and various antiviral agents can be tested for efficacy in central nervous system (CNS) infections.

Particles. Particles, including beads, liposomes and the like, constitute another entity useful in the practice of the present invention. Due to their larger size, particles may be conjugated with a larger number of light-generating molecules than, for example, can small molecules. This results in a higher concentration of light emission, which can be detected using shorter exposures or through thicker layers of tissue. In addition, liposomes can be constructed to contain an essentially pure targeting moiety, or ligand, such as an antigen or an antibody, on their surface. Further, the liposomes may be loaded with, for example, bioluminescent protein molecules, to relatively high concentrations (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)).

Furthermore, two types of liposomes may be targeted to the same cell type such that light is generated only when both are present. For example, one liposome may carry luciferase, while the other carries luciferin. The liposomes may carry targeting moieties, and the targeting moieties on the two liposomes may be the same or different. Viral proteins on infected cells can be used to identify infected tissues or organs. Cells of the immune system can be localized using a single or multiple cell surface markers.

The liposomes are preferably surface-coated, e.g., by incorporation of phospholipid—polyethyleneglycol conjugates, to extend blood circulation time and allow for greater targeting via the bloodstream. Liposomes of this type are well known.

Cells. Cells, both prokaryotic and eukaryotic, constitute another entity useful in the practice of the present invention. Like particles, cells can be loaded with relatively high concentrations of light-generating moieties, but have the advantage that the light-generating moieties can be provided by, for example, a heterologous genetic construct used to transfect the cells. In addition, cells can be selected that express "targeting moieties", or molecules effective to target them to desired locations within the subject. Alternatively, the cells can be transfected with a vector construct expressing an appropriate targeting moiety.

The cell type used depends on the application. For example, as is detailed below, bacterial cells, such as Salmonella, can be used to study the infective process, and to evaluate the effects of drugs or therapeutic agents on the infective process with a high level of temporal and spatial resolution.

Bacterial cells constitute effective entities. For example, they can be easily transfected to express a high levels of a light-generating moiety, as well as high levels of a targeting protein. In addition, it is possible to obtain *E. coli* libraries containing bacteria expressing surface-bound antibodies which can be screened to identify a colony expressing an antibody against a selected antigen (Stratagene, La Jolla, Calif.). Bacteria from this colony can then be transformed with a second plasmid containing a gene for a light-generating protein, and transformants can be utilized in the methods of the present invention, as described above, to localize the antigen in a mammalian host.

Pathogenic bacteria can be conjugated to a light-generating moiety and used in an animal model to follow the infection process in vivo and to evaluate potential anti-infective drugs, such as new antibiotics, for their efficacy in inhibiting the infection. An example of this application is illustrated by experiments performed in support of the present invention and detailed below.

Eukaryotic cells are also useful as entities in aspects of the present invention. Appropriate expression vectors, containing desired regulatory elements, are commercially available. The vectors can be used to generate constructs capable of expressing desired light-generating proteins in a variety of eukaryotic cells, including primary culture cells, somatic cells, lymphatic cells, etc. The cells can be used in transient expression studies, or, in the case of cell lines, can be selected for stable transformants.

Expression of the light-generating protein in transformed cells can be regulated using any of a variety of selected promoters. For example, if the cells are to be used as light-emitting entities targeted to a site in the subject by an expressed ligand or receptor, a constitutively-active promoter, such as the CMV or SV40 promoter may be used. Cells transformed with such a construct can also be used to assay for compounds that inhibit light generation, for example, by killing the cells.

Alternatively, the transformed cells may be administered such they become uniformly distributed in the subject, and express the light-generating protein only under certain conditions, such as upon infection by a virus or stimulation by a cytokine. Promoters that respond to factors associated with these and other stimuli are known in the art. In a related aspect, inducible promoters, such as the Tet system (Gossen and Bujard, 1992, *PNAS* 89:5547–5551) can be used to transiently activate expression of the light-generating protein.

For example, CD4+ lymphatic cells can be transformed with a construct containing tat-responsive HIV LTR elements, and used as an assay for infection by HIV (Israel and Honigman, 1991, *Gene* 104:139–145). Cells transformed with such a construct can be introduced into SCID-hu mice (McCune, et al., 1988, *Science* 241:1632–1639) and used as model for human HIV infection and AIDS.

Tumor cell lines transformed as above, for example, with a constitutively-active promoter, may be used to monitor the growth and metastasis of tumors. Transformed tumor cells may be injected into an animal model, allowed to form a tumor mass, and the size and metastasis of the tumor mass monitored during treatment with putative growth or metastasis inhibitors.

Tumor cells may also be generated from cells transformed with constructs containing regulatable promoters, whose activity is sensitive to various infective agents, or to therapeutic compounds.

Cell Transformation. Transformation methods for both prokaryotic cells and eukaryotic cells are well known in the art (Sambrook, et al., 1989, *In Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Vol. 2). Vectors containing the appropriate regulatory elements and multiple cloning sites are widely commercially available (e.g., Stratagene, La Jolla, Calif., Clontech, Palo Alto, Calif.).

D. Transgenic Animals Containing Genes Encoding Light-Generating Proteins

In another aspect, the present invention includes transgenic animals containing a heterologous gene construct encoding a light-generating protein or complex of proteins. The construct is driven by a selected promoter, and can include, for example, various accessory proteins required for the functional expression of the light-generating protein, as well as selection markers and enhancer elements.

Activation of the promoter results in increased expression of the genes encoding the light-generating molecules and accessory proteins. Activation of the promoter is achieved by the interaction of a selected biocompatible entity, or parts of the entity, with the promoter elements. If the activation occurs only in a part of the animal, only cells in that part will express the light-generating protein.

For example, an interferon-inducible promoter, such as the promoter for 3'-5' poly-A synthetase or the Mx protein (an interferon-inducible promoter), can be used to detect the infection of transgenic cells by a number of different RNA viruses.

In a related aspect, a promoter expressed in certain disease states can be used to mark affected areas in a transgenic animal, and expression of the light-generating moiety can be used to monitor the effects of treatments for the disease state. For example, E-selectin is expressed at sites of inflammation in vivo (Pober and Cotran, 1991, *Lab. Invest.* 64:301–305). Accordingly, the E-selectin promoter can be isolated and used to drive the expression of a luciferase gene.

It is also possible to use methods of the invention with tissue-specific promoters. This enables, for example, the screening of compounds which are effective to inhibit pathogenic processes resulting in the degeneration of a particular organ or tissue in the body, and permits the tracking of cells (e.g., neurons) in, for example, a developing animal.

Many promoters which are applicable for use with the present invention are known in the art. In addition, methods are known for isolating promoters of cloned genes, using information from the gene's cDNA to isolate promoter-containing genomic DNA.

In a specific embodiment of the present invention, transgenic animals expressing luciferase under the control of the HIV-1 LTR have been generated. As demonstrated in specific examples, luciferase expression serves as a real-time bioluminescent reporter which allows the noninvasive assessment of the level of promoter activity in vivo. As described, supra, the photons from the in vivo luciferase reaction can be detected by a CCD camera, after transmission through animal tissues, and used as an indication of the level and location of gene expression both in superficial and internal tissues.

E. Imaging Of Light-Emitting Conjugates

Light emitting conjugates that have localized to their intended sites in a subject may be imaged in a number of ways. Guidelines for such imaging, as well as specific examples, are described below.

1. Localization Of Light-Emitting Conjugates

In the case of "targeted" entities, that is, entities which contain a targeting moiety—a molecule or feature designed to localize the entity within a subject or animal at a particular site or sites, localization refers to a state when an equilibrium between bound, "localized", and unbound, "free" entities within a subject has been essentially achieved. The rate at which such an equilibrium is achieved depends upon the route of administration. For example, a conjugate administered by intravenous injection to localize thrombi may achieve localization, or accumulation at the thrombi, within minutes of injection. On the other hand, a conjugate administered orally to localize an infection in the intestine may take hours to achieve localization.

Alternatively, localization may simply refer to the location of the entity within the subject or animal at selected time periods after the entity is administered. For example, in experiments detailed herein, Salmonella are administered (e.g., orally) and their spread is followed as a function of time. In this case, the entity can be "localized" immediately following the oral introduction, inasmuch as it marks the initial location of the administered bacteria, and its subsequent spread or recession (also "localization") may be followed by imaging.

In a related aspect, localization of, for example, injected tumors cells expressing a light-generating moiety, may consist of the cells colonizing a site within the animal and forming a tumor mass.

By way of another example, localization is achieved when an entity becomes distributed following administration. For example, in the case of a conjugate administered to measure the oxygen concentration in various organs throughout the subject or animal, the conjugate becomes "localized", or informative, when it has achieved an essentially steady-state of distribution in the subject or animal.

In all of the above cases, a reasonable estimate of the time to achieve localization may be made by one skilled in the art. Furthermore, the state of localization as a function of time may be followed by imaging the light-emitting conjugate according to the methods of the invention.

2. Photodetector Devices

An important aspect of the present invention is the selection of a photodetector device with a high enough sensitivity to enable the imaging of faint light from within a mammal in a reasonable amount of time, preferably less than about 30 minutes, and to use the signal from such a device to construct an image.

In cases where it is possible to use light-generating moieties which are extremely bright, and/or to detect light-emitting conjugates localized near the surface of the subject or animal being imaged, a pair of "night-vision" goggles or a standard high-sensitivity video camera, such as a Silicon Intensified Tube (SIT) camera (e.g., Hamamatsu Photonic Systems, Bridgewater, N.J.), may be used. More typically, however, a more sensitive method of light detection is required.

In extremely low light levels, such as those encountered in the practice of the present invention, the photon flux per unit area becomes so low that the scene being imaged no longer appears continuous. Instead, it is represented by individual photons which are both temporally and spatially distinct form one another. Viewed on a monitor, such an image appears as scintillating points of light, each representing a single detected photon.

By accumulating these detected photons in a digital image processor over time, an image can be acquired and constructed. In contrast to conventional cameras where the signal at each image point is assigned an intensity value, in photon counting imaging the amplitude of the signal carries no significance. The objective is to simply detect the presence of a signal (photon) and to count the occurrence of the signal with respect to its position over time.

At least two types of photodetector devices, described below, can detect individual photons and generate a signal which can be analyzed by an image processor.

Reduced-Noise Photodetection Devices. The first class constitutes devices which achieve sensitivity by reducing the background noise in the photon detector, as opposed to amplifying the photon signal. Noise is reduced primarily by cooling the detector array. The devices include charge coupled device (CCD) cameras referred to as "backthinned", cooled CCD cameras. In the more sensitive instruments, the cooling is achieved using, for example, liquid nitrogen, which brings the temperature of the CCD array to approximately −120° C. The "backthinned" refers to an ultra-thin backplate that reduces the path length that a photon follows to be detected, thereby increasing the quantum efficiency. A particularly sensitive backthinned cryogenic CCD camera is the "TECH 512", a series 200 camera available from Photometrics, Ltd. (Tucson, Ariz.).

Photon Amplification Devices. A second class of sensitive photodetectors includes devices which amplify photons before they hit the detection screen. This class includes CCD cameras with intensifiers, such as microchannel intensifiers. A microchannel intensifier typically contains a metal array of channels perpendicular to and co-extensive with the detection screen of the camera. The microchannel array is placed between the sample, subject, or animal to be imaged, and the camera. Most of the photons entering the channels of the array contact a side of a channel before exiting. A voltage applied across the array results in the release of many electrons from each photon collision. The electrons from such a collision exit their channel of origin in a "shotgun" pattern, and are detected by the camera.

Even greater sensitivity can be achieved by placing intensifying microchannel arrays in series, so that electrons generated in the first stage in turn result in an amplified signal of electrons at the second stage. Increases in sensitivity, however, are achieved at the expense of spatial resolution, which decreases with each additional stage of amplification.

An exemplary microchannel intensifier-based single-photon detection device is the C2400 series, available from Hamamatsu.

Image Processors. Signals generated by photodetector devices which count photons need to be processed by an image processor in order to construct an image which can be, for example, displayed on a monitor or printed on a video printer. Such image processors are typically sold as part of systems which include the sensitive photon-counting cameras described above, and accordingly, are available from the same sources (e.g., Photometrics, Ltd., and Hamamatsu). Image processors from other vendors can also be used, but more effort is generally required to achieve a functional system.

The image processors are usually connected to a personal computer, such as an IBM-compatible PC or an Apple Macintosh (Apple Computer, Cupertino, Calif.), which may or may not be included as part of a purchased imaging system. Once the images are in the form of digital files, they can be manipulated by a variety of image processing programs (such as "ADOBE PHOTOSHOP", Adobe Systems, Adobe Systems, Mt. View, Calif.) and printed.

3. Immobilizing Subject In Detection Field Of Device

Detection Field Of Device. The detection field of the device is defined as the area from which consistent measurements of photon emission can be obtained. In the case of a camera using an optical lens, the detection field is simply the field of view accorded to the camera by the lens. Similarly, if the photodetector device is a pair of "night vision" goggles, the detection field is the field of view of the goggles.

Alternatively, the detection field may be a surface defined by the ends of fiber-optic cables arranged in a tightly-packed array. The array is constructed to maximize the area covered by the ends of the cables, as opposed to void space between cables, and placed in close proximity to the subject. For instance, a clear material such as plexiglass can be placed adjacent the subject, and the array fastened adjacent the clear material, opposite from the subject.

The fiber-optic cable ends opposite the array can be connected directly to the detection or intensifying device, such as the input end of a microchannel intensifier, eliminating the need for a lens.

An advantage of this method is that scattering and/or loss of photons is reduced by eliminating a large part of the air space between the subject and the detector, and/or by eliminating the lens. Even a high-transmission lens, such as the 60 mm AF Nikkor macro lens used in experiments performed in support of the present invention, transmits only a fraction of the light reaching the front lens element.

With higher-intensity LGMs, photodiode arrays may be used to measure photon emission. A photodiode array can be incorporated into a relatively flexible sheet, enabling the practitioner to partially "wrap" the array around the subject. This approach also minimizes photon loss, and in addition, provides a means of obtaining three-dimensional images of the bioluminescence.

Other approaches may be used to generate three-dimensional images, including multiple detectors placed around the subject or a scanning detector or detectors.

It will be understood that the entire animal or subject need not necessarily be in the detection field of the photodetection device. For example, if one is measuring a light-emitting conjugate known to be localized in a particular region of the subject, only light from that region, and a sufficient surrounding "dark" zone, need be measured to obtain the desired information.

Immobilizing The Subject. In those cases where it is desired to generate a two-dimensional or three-dimensional image of the subject, the subject may be immobilized in the detection field of the photodetection devices during the period that photon emission is being measured. If the signal is sufficiently bright that an image can be constructed from photon emission measured in less than about 20 milliseconds, and the subject is not particularly agitated, no special immobilization precautions may be required, except to insure that the subject is in the field of the detection device at the start of the measuring period.

If, on the other hand, the photon emission measurement takes longer than about 20 msec, and the subject is agitated, precautions to insure immobilization of the subject during photon emission measurement, commensurate with the degree of agitation of the subject, need to be considered to preserve the spatial information in the constructed image. For example, in a case where the subject is a person and photon emission measurement time is on the order of a few seconds, the subject may simply be asked to remain as still as possible during photon emission measurement (imaging). on the other hand, if the subject is an animal, such as a mouse, the subject can be immobilized using, for example, an anesthetic or a mechanical restraining device.

A variety of restraining devices may be constructed. For example, a restraining device effective to immobilize a mouse for tens of seconds to minutes may be built by fastening a plexiglass sheet over a foam cushion. The cushion has an indentation for the animal's head at one end. The animal is placed under the plexiglass such that its head

is over the indentation, allowing it to breathe freely, yet the movement of its body is constrained by the foam cushion.

In cases where it is desired to measure only the total amount of light emanating from a subject or animal, the subject does not necessarily need to be immobilized, even for long periods of photon emission measurements. All that is required is that the subject be confined to the detection field of the photodetector during imaging. It will be appreciated, however, that immobilizing the subject during such measuring may improve the consistency of results obtained, because the thickness of tissue through which detected photons pass will be more uniform from animal to animal.

4. Further Considerations During Imaging

Fluorescent Light-Generating Moieties. The visualization of fluorescent light-generating moieties requires an excitation light source, as well as a photodetector. Furthermore, it will be understood that the excitation light source is turned on during the measuring of photon emission from the light-generating moiety.

Appropriate selection of a fluorophore, placement of the light source and selection and placement of filters, all of which facilitate the construction of an informative image, are discussed above, in the section on fluorescent light-generating moieties.

High-Resolution Imaging. Photon scattering by tissue limits the resolution that can be obtained by imaging LGMs through a measurement of total photon emission. It will be understood that the present invention also includes embodiments in which the light-generation of LGMs is synchronized to an external source which can be focused at selected points within the subject, but which does not scatter significantly in tissue, allowing the construction of higher-resolution images. For example, a focused ultrasound signal can be used to scan, in three dimensions, the subject being imaged. Light-generation from areas which are in the focal point of the ultrasound can be resolved from other photon emission by a characteristic oscillation imparted to the light by the ultrasound (e.g., Houston and Moerner, U.S. Pat. No. 4,614,116, issued Sep. 30, 1986.)

5. Constructing An Image Of Photon Emission

In cases where, due to an exceptionally bright light-generating moiety and/or localization of light-emitting conjugates near the surface of the subject, a pair of "night-vision" goggles or a high sensitivity video camera was used to obtain an image, the image is simply viewed or displayed on a video monitor. If desired, the signal from a video camera can be diverted through an image processor, which can store individual video frames in memory for analysis or printing, and/or can digitize the images for analysis and printing on a computer.

Alternatively, if a photon counting approach is used, the measurement of photon emission generates an array of numbers, representing the number of photons detected at each pixel location, in the image processor. These numbers are used to generate an image, typically by normalizing the photon counts (either to a fixed, pre-selected value, or to the maximum number detected in any pixel) and converting the normalized number to a brightness (greyscale) or to a color (pseudocolor) that is displayed on a monitor. In a pseudocolor representation, typical color assignments are as follows. Pixels with zero photon counts are assigned black, low counts blue, and increasing counts colors of increasing wavelength, on up to red for the highest photon count values. The location of colors on the monitor represents the distribution of photon emission, and, accordingly, the location of light-emitting conjugates.

In order to provide a frame of reference for the conjugates, a greyscale image of the (still immobilized) subject from which photon emission was measured is typically constructed. Such an image may be constructed, for example, by opening a door to the imaging chamber, or box, in dim room light, and measuring reflected photons (typically for a fraction of the time it takes to measure photon emission). The greyscale image may be constructed either before measuring photon emission, or after.

The image of photon emission is typically superimposed on the greyscale image to produce a composite image of photon emission in relation to the subject.

If it desired to follow the localization and/or the signal from a light-emitting conjugate over time, for example, to record the effects of a treatment on the distribution and/or localization of a selected biocompatible moiety, the measurement of photon emission, or imaging can be repeated at selected time intervals to construct a series of images. The intervals can be as short as minutes, or as long as days or weeks.

F. Analysis Of Photon Emission Images

Images generated by methods and/or using compositions of the present invention may be analyzed by a variety of methods. They range from a simple visual examination, mental evaluation and/or printing of a hardcopy, to sophisticated digital image analysis. Interpretation of the information obtained from an analysis depends on the phenomenon under observation and the entity being used.

The following experiments illustrate one application of the present invention—tracking Salmonella infection in live mice—and how images obtained using methods of the present invention can be analyzed. Similarily, infection of numerous other pathogens, including, but not limited to, Pseudomonas, Staphylococcus, Streptococcus, Enterococcus, Enterobacter, Citrobacter, Leginella, Helicobacter, Acinetobacter, Escherichia, Klebsiella and Serratia.

G. Imaging Of Luminescent Salmonella In Living Mice

Experiments performed in support of the present invention characterize the distribution of *Salmonella typhimurium* infection in mice, the animal model of human typhoid. A mouse virulent *Salmonella typhimurium* strain, SL1344 (Hoiseth and Stocker, 1981, *Nature* 291:238–239), a non-invasive mutant of SL1344, BJ66 and a low virulence LT-2 strain of Salmonella, LB5000 were each marked with a plasmid containing the lux operon, and used in experiments to localize Salmonella infection in mice.

1. Constructions Of Luminescent Salmonella

Salmonella Strains. Three strains of *Salmronella typhimurium* with differing virulence phenotypes, defined by oral and intra-peritoneal inoculations into mice, are selected for transformation.

The most virulent phenotype used herein is SL1344, a mouse strain originally obtained from a fatal infection of a calf (Hoiseth and Stocker, 1981, *Nature* 291:238–239). Following oral inoculations of mice with this strain, bacteria are disseminated systematically via the lymphatic system resulting in colonization of the liver, spleen and bone marrow (Carter and Collins, 1974, *J. Exper. Med.* 139:1189–1203.; see also reviews by Finlay and Falkow, 1989, *Mol. Microbiol.* 3:1833–1841, and Hsu, 1989, *Microbiol. Rev.* 53:390–409.)

A non-invasive mutant of SL1344, BJ66, is also evaluated. Systemic infections in mice do not typically result from an oral inoculation with BJ66, but do result from intraperitoneal inoculations with this strain.

A low virulence LT-2 strain of Salmonella, LB5000, is also examined. LT-2 stains are laboratory strains known to be of reduced or variable virulence for mice. LB5000 contains multiple auxotrophic mutations, is streptomycin resistant, and is cleared from mice following oral or intraperitoneal inoculations.

Transformation of Salmonella Strains with the lux Operon. The three strains are each transformed with a plasmid encoding the lux operon, as detailed in Example 1. The plasmid, obtained from the soil bacterium *Xenorhabdus luminescens* (Frackman, et al., 1990) confers on *E coli* the ability to emit photons through the expression of the two subunits of the heterodimeric luciferase and three accessory proteins, luxC, luxD and luxE.

Inclusion of luxC, luxD and luxE removes the necessity of providing the fatty aldehyde substrate, luciferin, to the luciferase-expressing cells. Because supplying the substrate to eukaryotic luciferase enzymes in an in vivo system such as described herein may prove difficult, the entire lux operon of *X. luminescens* is used. The operon also encodes the enzymes for the biosynthesis of the fatty aldehyde substrate.

X. luminescens luciferase, an alpha-beta heterodimeric mixed-function oxidase, catalyzes the oxidation of reduced flavin and long-chain aldehyde to oxidized flavin and the corresponding long-chain fatty acid. A fatty acid reductase complex is required for the generation and recycling of fatty acid to aldehyde, and an NAD(P)H:flavin oxidoreductase supplies the reduced flavin.

Optimal bioluminescence for *E. Coli* expressing the lux genes of *X. luminescens* is 37° C. (Szittner and Meighen, 1990, *J. Biol. Chem.* 265:16581–16587, Xi, et al., 1991, *J. Bact.* 173:1399–1405). In contrast, luciferases from eukaryotic and other prokaryotic luminescent organisms typically have lower temperature optima (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). The luciferase from *X. lumiiescens*, therefore, is well-suited for use as a marker for studies in animals.

The three strains are transformed by electroporation with the plasmid pGSL1, which contains the entire *X. luminescens* lux operon and confers resistance to ampicillin and carbenicillin on the Salmonella (Frackman, et al., 1990). The *X. luminescens* lux operon contains the genes luxA, luxB, luxC, luxD and luxE (Frackman, et al., 1990). LuxA and B encode the two subunits of the heterodimeric luciferase. luxc and D encode the biosynthetic enzymes for the luciferase substrate and luxE is a regulatory gene. Inclusion of the genes for the biosynthesis of the substrate is a convenient means of providing substrate to luciferase, in contrast to supplying luciferin externally to the cells in culture or treating animals with the substrate.

2. Characterization Of Transformed Salmonella In Vitro

Adherence And Invasive Properties. The adherence and invasive properties of the three Salmonella strains containing the lux plasmid are compared in culture, to each other, and to their non-luminescent parental strains by the standard invasion assay as described by Finlay and Falkow, 1989, *Mol. Microbiol.* 3:1833–1841., and detailed in Example 2.

In this assay, adherent and intracellular bacteria are quantified following incubation with an epithelial cell line and peritoneal macrophages. The adherent and intracellular bacteria are detected and quantified by both the emission of photons from living cells, and colony forming units following lysis and plating the cell lysates on carbenicillin-containing plates.

The results of some of the assays are shown in FIGS. 2A through 2E and discussed in Example 8. The phenotypes of the three strains transformed with the lux expressing plasmid are not significantly altered in comparison to the parental Salmonella strains. In addition, there is a good correlation between the intensity of bioluminescence and the CFU from the HEp-2 cells and macrophages. The results show that luminescence, as an indicator of intracellular bacteria, is a rapid method for assaying the invasive properties of bacteria in culture.

BJ66 demonstrated reduced adherence to HEp-2 cells in comparison to SL1344, however, adherence of the two strains in primary cultures of murine peritoneal macrophages were comparable.

Light Emission. To evaluate the oxygen requirements of the system, 10 fold serial dilutions of bacteria are placed in glass capillary tubes and imaged, as detailed in Example 3.

FIG. 3 shows an image generated in one such experiment. Luminescence is only detected at the air-liquid interface, even in the tubes with small numbers of bacteria in air saturated medium (0.1 ml of air saturated buffer in 5 l results in a final O_2 concentration of 5 nM).

From these results, it is apparent that oxygen is likely a limiting factor for luminescence.

Light Transmission Through Animal Tissue. To determine the degree to which light penetrates animal tissue, light emitted from luminescent Salmonella and transmitted through tissue is quantified using a scintillation counter, with the fast coincidence detector turned off to detect single photons. The background due to dark current of the photomultiplier tubes in this type of detection is significant, limiting the assay to samples with relatively strong photon emission.

Four tissue types of varying opacity are compared using this approach: muscle from chicken breast, skin from chicken breast, lamb kidney and renal medulla from lamb kidney. The number of photons that can be detected through tissue is approximately ten fold less than the controls without tissue.

3. Characterization Of Lux Salmonella In Vivo

Oral Administration. Oral inoculation is natural route of infection of mice or humans with Salmonella and results in a more protracted course of disease. In order to study the progression of the Salmonella infection following this route of inoculation, two strains of mice are infected with the three strains of Salmonella. The results obtained using the resistant animals are discussed under the heading "Infection of Resistant Mice", below.

Balb/c mice are orally infected with suspensions of virulent SL1344lux, non-invasive BJ66lux and low virulence LB5000lux Salmonella, as described in Example 5. Progression of the infection is followed by external imaging (Materials and Methods) over an 8 day period.

Representative images are shown in FIGS. 6A, 6B, and 6C. At 24 hours post inoculation (p.i.), the bioluminescent signal is localized at a single focus in all infected animals (FIGS. 6A, 6B and 6C). Bioluminescence disappears in all animals infected with the low virulence LB5000lux by 7 days p.i. (FIG. 6A). Animals infected with the virulent SL1344lux, on the other hand, show virulent infection which often spreads over much of the abdominal cavity (FIG. 6C), though the time at which it begins to spread is highly variable from animal to animal. The infection by BJ66lux typically persists and remains localized at a single site (FIG. 6B).

I.P. Inoculation. To assess whether or not there is sufficient O_2 at the sites of Salmonella replication for the oxidation of luciferin and subsequent luminescence (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)), photon emission is measured from the tissues of a respiring animal. Luminescent SL1344lux and LB5000lux are inoculated into the peritoneal cavities of two groups of Balb/c mice. 32 hours post inoculation (p.i.), the transmitted photons are imaged (FIG. 7).

In the mice infected with SL1344lux (left part of FIGURE), transmitted photons are evident over a large surface, with foci of varying intensities visible. These images are indicative of a disseminated infection, and are consistent with widespread colonization of the viscera, possibly including the liver and mesenteric lymph nodes. In contrast, the distributions of transmitted photons from animals infected with the LB5000lux strain is very limited, indicating a limited infection.

The LB5000lux-infected mice remained healthy for several weeks p.i., while the SL1344lux-infected mice were nearly moribund and euthanized at 4 days p.i.

These experiments indicate that the level of 02 in the blood and or tissues is adequate for bioluminescence of lux luciferase expressed by Salmonella. Furthermore, the experiments are consistent with the invasive nature of the virulent strain SL1344 in comparison to the reduced virulent laboratory strain LB5000.

Infection Of Resistant Mice. Mice which are heterozygous at the Ity locus ($Ity^{r/s}$) are resistant to systemic infections by *S. typhimurium* (Plant and Glynn, 1976, *J. Infect. Dis.* 133:72–78). This locus, also called Bcg (Gros, et al., 1981, *J Immunol.* 127:2417–2421) or Lsh (Bradley, 1977, *Clin. and Exper. Immunol.* 30:130–140), regulates the pathogenic processes of certain intracellular pathogens, such as *Mycobacterium lepraemurium* (Forget, et al., 1981, *Infect. Immunol.* 32:42–47), M. Bovis (Skamene, et al., 1984, *Immunogenet.* 19:117–120, Skamene and Pietrangeli, 1991, *Nature* 297:506–509) and M. intracelluare (Goto, et al., 1989, *Immunogenetics* 30:218–221). An analogous genetic control of resistance and susceptibility to intracellular pathogens appears to be in humans as well (*M. tuberculosis* (Stead, 1992, *Annals of Intern. Med.* 116:937–941, Stead, et al., et al., 1990, *New Eng. J. Med.* 322:422–427) and *M. leprae*).

The Ity locus is located on mouse chromosome 1 with two allelic forms, Ity^r (resistant, dominant) and Ity^s (sensitive, recessive). The gene encoded at the Ity locus apparently affects the ability of macrophages to disrupt the internalized pathogens (reviewed by Blackwell, et al., 1991, *Immunol. Lett.* 30:241–248 (1991); see also Skamene, et al., 1984, *Immunogenet.* 19:117–120, Skamene and Pietrangeli, 1991, *Nature* 297:506–509) which in turn, affects the down stream function of the proposed macrophage-mediated transport of pathogens to other sites within the infected host. Balb\c mice are $Ity^{s/s}$ and 129 mice are $Ity^{r/r}$. The heterozygous Balb\cx 129 mice ($Ity^{r/s}$) are used in experiments detailed herein.

Resistant 129×Balb/c ($Ity^{r/s}$) viable mice are infected by intragastric inoculation of 1×10^7 SL1344lux Salmonella as detailed in Example 7. The animals are imaged daily for 8 days post injection (d.p.i.).

Results are shown in FIGS. 8A (day 1) and 8B (day 8). The luminescence, detected by external imaging, is apparent at 24 h p.i., and appeared to localized to a single site in all animals. The luminescent signal is present throughout the study period (up to 8 days p.i.). The intensity of the luminescence and the location of the luminescent source is somewhat variable over time within a mouse and also from mouse to mouse. The luminescent tissue in all infected animals is the cecum (see below) and the variability in localization, and possibly intensity, is most likely due fact that internal organs of rodents are not tightly fixed in position.

The apparent limited infection observed in these animals supports the interpretation that the Ity restriction blocks macrophage transport. The persistence of this infection for 10 days, however, suggests that there is adherence to the intestine mucosa and prolonged shedding of bacteria in the feces of these animals, as evidenced by luminescent fecal pellets. These results indicate that the luminescent phenotype of the Salmonella in vivo is retained over an 8 day durazion in Ity restricted animals and that localization is possible following an oral inoculation.

Internal Imaging Following Oral Inoculation. In order to further localize the luminescent signal in the abdominal cavity, infected mice are imaged following laparotomy (Example 8). The predominant disease manifestation in all of the animals infected by the oral route is an enlarged cecum (FIGS. 9A, 9B, 9C). The "external" image (FIG. 9A) illustrates a focal luminescence, which is revealed in the post-laparotomy image (FIG. 9B) to be the cecum.

Injection of air into the intestine confirms the presence of bacteria in other regions of the digestive tract. Bacteria in the colon and rectum are likely expressing luciferase, but low oxygen concentrations are likely limiting light emission from these sites.

The images obtained from oral inoculation studies indicate that the luminescent signal, at 2 days p.i. and at 7 days p.i., localizes almost entirely to the cecum in each of the animals (Popesko, et al., 1990, *A Colour Atlas of Anatomy of Small Laboratory Animals Vol. Two: Rat Mouse Hamster* (London England: Wolfe)) except those infected with LB5000lux. Luminescence is also apparent in the colon in some animals. By 7 days p.i., no luminescence is detectable in the LB5000lux-infected animals. The CFU present in the organs of these mice are determined at 2 and 5 d p.i.

In animals infected intragastrically with the invasive strain, SL1344lux, the luminescence in the cecum appears early and precedes a systemic infection. In contrast, infections with the non-invasive BJ66lux strain result in a persistent luminescence from the cecum that remains, in some animals, for the entire course of the study (8 days). By 8 days p.i., luminescence is detected over much of the abdominal surface, resembling the distribution of photons following an i.p. inoculation, in the SL1344lux infected mice.

Infections with SL1344lux appear to become systemic, as predicted, with progressively more photons being emitted from an increasing surface area. Luminescence appears to localize over the abdomen in infections with all strains with little detectable luminescence from outside this area. A large number of transmitted photons are localized as a single focus over the abdomen suggesting that even though the infection may be systemic, the greatest amount of replication may be in areas surrounding the intestine.

Localization of the luminescence over the cecum indicates that not only are there large numbers of organisms in this region of the intestine, but also suggests that the Salmonella associate with cells of the mucosa such that they can obtain sufficient oxygen for luminescence. Emission of photons from luciferase is oxygen dependent and the expected oxygen levels in the lumen of the cecum, or

intestine in general, are below the levels required for luminescence. The luciferase reaction is not expected to be functional in the intestine unless the bacteria can obtain oxygen from cells of the intestinal epithelium.

Thus, the systemic infection seems to be related to the invasive phenotype and not to simply adherence to epithelial cells of the intestine. These experiments implicate the cecum in some role in the pathogenic process either in the carrier state or as a site of dissemination.

Monitoring the progression of infections to different tissues may greatly enhance the ability to understand these steps in the pathogenic process, and enable the screening for compounds effective to inhibit the pathogen at selected steps.

Internal Imaging Following I.P. Inoculation. Mice infected intraperitoneally with SL1344lux are imaged before and after laparotomy (Example 9). The results are shown in FIG. **10**. The images demonstrate luminescence over a majority of the abdomen with multiple foci of transmitted photons. The cecum does not appear to contain luminescent Salmonella. The results from these experiments indicate that all strains of Salmonella have sufficient 02 to be luminescent in the early phases of infection. However, entry of Salmonella into cells of the mucosa and subsequent systemic infection is likely limited to strains with the invasive phenotype, since systemic infections at later time points are only apparent in SL1344lux-infected mice.

Effects Of Ciprofloxacin On Salmonella Infection. Experiments, detailed in Example 10, are performed to demonstrate that non-invasive imaging is useful for following the response of an infection to drugs. Mice are orally inoculated with SL1344lux and treated with 100 mg of ciprofloxacin, an antibiotic effective against Salmonella infections. The mice are imaged at selected time periods following treatment, and the extent of infection is quantitated by measuring photon emission. Photon emission in treated mice is compared to values before the initiation of treatment, and to values from control mice that had been infected, but not treated. Results from one such experiment are shown in FIGS. **11A**, **11B**, **11C**, **11D**, and **11E** and discussed in Example 10. Infection is significantly reduced in mice treated with the antibiotic, compared both to the levels of pathogen at time zero in treated animals, and to levels of pathogen in control animals throughout the treatment period.

Effects Of Carbenenicillin Selection. Ducluzeau, et al., 1970, *Zeut. Bakt.* 5313:533–548., demonstrated that treatment of animals with antibiotics facilitated colonization of the cecum with Salmonella. The mice in the present experiments are maintained on an antibiotic regime of intramuscular injections of carbenicillin for the purpose of selecting the Amp^r Salmonella containing the luciferase clone. This treatment may alter the course of the gastrointestinal infection, but the observation that Salmonella can associate with the cells lining the cecum indicates that oxygen is available for luminescence. This observation is notable, since the lumen of the cecum is commonly thought to be an anaerobic environment.

H. Applications

The bioluminescence technology is broadly applicable to a variety of hostpathogen systems and may also enable temporal and spatial evaluation of other biological events, as for example tumor progression and gene expression in living mammals, and have application in pharmaceutical development and screening. Widespread use of in vivo imaging of pathogens may reduce the numbers of animals and time needed for experiments pertaining to pathogenesis and/or the real-time study antimicrobial agents. Furthermore, bioluminescent organisms may be useful as biosensors in the living animal, much as luminescent bacteria are used in environmental analyses. Korpela et al., for example, demonstrate that the limited oxygen supply in the lumen of the G.I. tract restricted bioluminescence to sites in which oxygen is accessible to the Salmonella, perhaps directly from epithelial or other cell types. Korpela, et al., 1989, *J. Biolum. Chemilum.* 4:551–554. This oxygen requirement may find utility as an indicator of intimate cell—cell interactions, or as a biosensor for studying oxygen concentrations at various sites in living animals. In the following, several exemplary applications of this technology are described for the purpose of illustration, but are in no way intended to limit the present invention.

1. Determination Of Oxygen Levels

The oxygen requirement for luminescence of luciferase evidenced in the experiments summarized above indicates that the present invention may be applicable as a method of determining spatial gradients of oxygen concentration in a subject. Luminescent bacteria have been used to measure oxygen levels in the range of 10–1 mM. The studies predict that 0.1 nM is the lower limit of detection (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). The imaging methods described herein may be used for studying oxygen levels at various sites in living animals. For example, microorganisms that have been engineered to emit light in an O_2 or Ca^{2+}-dependent manner could be used as biosensors in a subject, much like luminescent bacteria are used in environmental analyses (Guzzo, et al., 1992, *Tox. Lett.* 64/65:687–693, Korpela, et al., 1989, *J. Biolum. Chemilum.* 4:551–554, Jassim, et al., 1990, *J. Biolum. Chemilum.* 5:115–122). The dynamic range of luminescence with respect to O_2 concentration is much broader and reaches lower O_2 concentrations than O_2 probes (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). Moreover, light emission in proportion to O_2 concentration is linear over a range of 30 nM to 8 mM, and 9 mM O_2 is required for ½ maximal luminescence.

2. Localization Of Tumor Cells

The growth and metastatic spread of tumors in a subject may be monitored using methods and compositions of the present invention. In particular, in cases where an individual is diagnosed with a primary tumor, LECs directed against the cells of the tumor can be used to both define the boundaries of the tumor, and to determine whether cells from the primary tumor mass have migrated and colonized distal sites.

For example, LECs, such as liposomes containing antibodies directed against tumor antigens and loaded with LGMs, can be administered to a subject, allowed to bind to tumor cells in the subject, imaged, and the areas of photon emission can be correlated with areas of tumor cells.

In a related aspect, images utilizing tumor-localizing LECs, such as those described above, may be generated at selected time intervals to monitor tumor growth, progression and metastasis in a subject over time. Such monitoring may be useful to record results of anti-tumor therapy, or as part of a screen of putative therapeutic compounds useful in inhibiting tumor growth or metastasis.

Alternatively, tumor cells can be transformed, transduced, transiently or permanently, or otherewisw made to emit

light, with a luciferase construct under the control of a constitutively-active promoter, and used to induce luminescent tumors in animal models, as described above. Such animal models can be used for evaluating the effects of putative anti-tumor compounds.

3. Localization Of Inflammation

In an analogous manner to that described above, compositions and methods of the present invention may be used to localize sites of inflammation, monitor inflammation over time, and/or screen for effective anti-inflammatory compounds. Molecules useful for targeting to sites of inflammation include the ELAN family of proteins, which bind to selections. An ELAN molecule can be incorporated as a targeting moiety on an entity of the present invention, and used to target inflammation sites.

Alternatively, an animal model for the study of putative anti-inflammatory substances can be made by making the animal transgenic for luciferase under the control of the E-selectin promoter. Since E-selectin is expressed at sites of inflammation, transgenic cells at sites of inflammation would express luciferase.

The system can be used to screen for anti-inflammatory substances. Inflammatory stimuli can be administered to control and experimental animals, and the effects of putative anti-inflammatory compounds evaluated by their effects on induced luminescence in treated animals relative to control animals.

4. Localization Of Infection

As illustrated in experiments performed in support of the present invention and summarized above, LGCs may be effectively used to follow the course of infection of a subject by a pathogen, including, but not limited to, Pseudomonas, Staphylococcus, Streptococcus, Enterococcus, Enterobacter, Citrobacter, Leginella, Helicobacter, Acinetobacter, Escherichia, Klebsiella or Serratia. In experiments detailed herein, the LGCs are pathogenic cells (Salmonella) transformed to express luciferase. Such a system is ideally-suited to the study of infection, and the subsequent spread of infection, in animal models of human diseases. It provides the ability to monitor the progression of an infectious disease using sites of infection and disease progression rather than traditional systemic symptoms, such as fever, swelling, etc. in studies of pathogenesis.

Use of an external imaging method to monitor the efficacy of anti-infectives permits temporal and spatial evaluations in individual living animals, thereby reducing the number of animals needed for experiments pertaining to pathogenesis and/or the study anti-infective agents.

5. Monitoring Promoter Activity In Transgenic Mice

The generation of transgenic animals has become an important tool in basic research and in the development of gene therapies and gene vaccines. The present invention provides methods for rapid in situ assessment of the uptake of nucleic acids and their expression and thus the evaluation of gene delivery systems and DNA-based therapies.

More specifically, luciferase expression may serve as a real-time bioluminescent reporter, allowing the noninvasive assessment of the level of promoter activity in living animals. Photons from the in vivo luciferase reaction in the transgenic animal are detected by a CCD camera, after transmission through animal tissues, and used as an indication of the level and location of gene expression. This way, a real-time assessment of the extent of promoter activity in both superficial and deep tissues can be accomplished.

As described in specific embodiments of the present invention, the light-emitting reporter systems in transgenic animals facilitate in vivo assessment of the regulation of gene expression, thus facilitating the development of novel therapies that target regulation of viral and host gene expression. Bioluminescent reporters offer the advantages of spontaneous emission of light without a need for outside light sources, low background signal permitting near single-event detection, real-time analyses, and the absence of cytotoxic photosensitizing dyes. As such, bioluminescent reporters have a greater versatility than fluorescent markers in mammalian tissues. Biological processes can be viewed in vivo by illuminating the temporal and spatial distribution of gene expression in animals and humans.

The in vivo monitoring of promoter activity as described herein can be used for the assessment of gene delivery and expression in gene therapies, gene vaccines, antisense oligonucleotide therapies, the generation of chimeric and transgenic animals in research. The technology is further useful for real-time noninvasive assays for gene expression in research environments involving questions of developmental regulation, response to infectious disease or other systems where gene expression demonstrates change.

The following examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. The present invention is explained in more detail by means of the below examples.

VI. EXAMPLES

A. Materials and Methods

1. Cells

Salmonella strains SL1344 and LB5000 were obtained from B. A. D. Stocker (Stanford University; Hoiseth and Stocker, 1981, *Nature* 291:238–239). Salmonella strain BJ66 was obtained from B. D. Jones (Stanford University).

HEp-2 cells were obtained from the American Type Culture Collection (ATCC; 12301 Parklawn Dr., Rockville Md.; Accession number CCL-23).

Murine peritoneal macrophages were obtained by peritoneal lavage of euthanized Balb/c mice with 7 ml of growth medium (Maximow and Bloom, 1931, *Textbook of Histology,* Saunders, Philadelphia.)

2. Static Cultures

Low oxygen (static) cultures were prepared by inoculating 3 ml of LB Broth containing 100 mg/ml of carbenicillin with 6 μl of a bacterial suspension from a stationary phase culture, and growing the bacteria at 37° C. overnight in a stationary 7 ml culture tube.

3. Mice

Balb/c ($It^{s/s}$) mice were obtained from the Department of Oncology, Stanford University. 129×Balb/c ($Ity^{r/s}$) mice were obtained from the Stanford Transgenic Animal Facility (Stanford, Calif.). All animals were housed under identical conditions of photo period, feeding regime and temperature in the Stanford University Research Animal Facility (Stanford, Calif.).

Anesthesia was performed by injecting the animals intraperitoneally (i.p.) with 33 pg/kg body weight nembutal.

Euthanasia was performed by asphyxiation in CO_2 or cervical dislocation, following protocols recommended by the Stanford University Research Animal Facility. Cervical dislocation was used in experiments in which results may have been affected by physiological changes due to asphyxia.

Mice infected with lux-transformed Salmonella were given daily intramuscular (i.m.) injections of carbenicillin (125 mg per kg body weight) to maintain selective pressure on the luminescent Salmonella for retention of the Amp^r plasmid containing the lux operon.

4. Imaging

Animals or objects to be imaged were immobilized in a light-tight box containing a door and a charge-coupled device (CCD) camera with a two stage microchannel intensifier head (model C2400-40, Hamamatsu). The camera was attached, via cables leading out of the box, to an "ARGUS 50" image processor (Hamamatsu).

The ICCD system described above is capable of detecting sinole photons once a threshold of 10–30 photons is achieved. The signal to noise ratio of the system ranged from 2:1 to 1×10^4:1, depending on signal intensity.

Grey-scale images were obtained by opening the light box door in dim room light and integrating for 8–64 frames. The gain for the gray scale images was set to optimize the image—typically at 3000 volts on a scale of 0 to 10,000 volts.

Bioluminescence data were obtained in absence of external illumination. Exposure settings were as follows: the black level was set automatically by the camera/image processor, the gain was adjusted automatically by the intensifier controller, and the f-stop was set at 2.8. A 60 mm "AF NIKKOR" macro lens was used (Nikon Inc., Melville, N.Y.).

Bioluminescence images were generated by integrating photons for a selected period of time, typically 5 minutes. Data are presented at the lowest bit range setting of 0–3 bits per pixel for all animals. For images of other objects, i.e., 24 well plates, where the resolution of the bioluminescent signals was not possible at a bit range of 0–3, the range was increased to a setting that permitted localization of bioluminescent signals, typically 1–7. Objects were imaged for shorter periods of time when additional information could not be obtained by imaging for five minutes.

External imaging refers to non-invasive imaging of animals. Internal imaging refers to imaging after a partial dissection of the animals, typically a laparotomy. Internal imaging is performed in selected animals to confirm the sources of photon emission localized by external imaging.

The bioluminescence image data are presented as a pseudo-color luminescence image representing the intensity of the detected photons. Six levels of intensity are typically used, ranging from blue (low intensity) to red (higher intensity).

To generate the FIGURES presented herein, greyscale and bioluminescence images were superimposed, using the image processor, to form a composite image providing a spatial frame of reference.

The composite image was displayed on an RGB CRT (red, green, blue; cathode ray tube) monitor, and the monitor was photographed to produce hardcopies. Hardcopies were also generated by saving the image processor image as a digital file, transferring the file to a computer, and printing it on a color printer attached to the computer. Alternatively, hardcopies may be generated by printing the video signal directly using a video printer.

B. Example 1

Transformation of Salmonella with pCGLS1 lux Plasmid

Salmonella strains SL1344, BJ66 and LB5000 were transformed with pCGLS1, a pUC!8-based vector encoding the lux operon from *Xenorhabdus luminescens* (Frackman, et al., 1993).

1. pCGLS1 Plasmid

A schematic of the pCGLS1 plasmid is shown in FIGS. 1A, 1B and 1C. The plasmid was constructed by cloning an ~11 kb region encoding the lux genes from the soil bacterium *Xenorhabdus luminescens* (FIG. 1A; Frackman, et al., 1990) into the Bam HI site (FIG. 1B) of pUC18 (FIG. 1C; Clontech, Palo Alto, Calif.). The construction of the vector is described by Frackman, et al., (1990).

Restriction enzyme sites in FIG. 1A are represented as follows: Bs, Bst EII; C, Cla I; E, Eco RI; H, Hind III; M, Mlu I; S, Sca I; X, Xba I; B/Sa, Bam HI and Sau 3A junction. A sequence included in the multiple cloning site (MCS) is provided in FIG. 1B, with the Bam HI site indicated in bold type.

A graphical representation of a pUC18 vector with no insert is shown in FIG. 1C. Labeled elements include an ampicillin resistance gene (Ap), a lac Z gene (lac Z) and an *E. coli* origin of replication (Ori). The unmodified pUC18 vector is approximately 2.7 kb in size.

2. Transformation Of Salmonella

Electrocompetent cells from Salmonella strains SL1344, BJ66 and LB5000 were made using standard methods (Sambrook, et al., 1989, *In Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Vol. 2) and stored at −80° C. until just prior to use. Electroporation was performed as follows: 1 μl of the plasmid (0.2 μg/ml) was added to 40 μl of ice-cold electrocompetent cells suspended in 10% glycerol. The suspension was mixed gently for one minute, placed in a 1 mm gap electroporation cuvette and electroporated using a Bio-Rad Gene-Pulser (Bio-Rad Laboratories, Hercules, Calif.). The settings were 2.5 kvolts, 400 ohms and 25 μfarads.

Following a one hour agitated incubation in Luria Bertini (LB) broth at 37° C., the cells were plated on (LB) Agar containing 100 μg/ml carbenicillin and allowed to grow overnight.

To maximize the bioluminescence of the labelled Salmonella, the lux operon was maintained on a high-copy-number plasmid and not integrated as a single copy gene. However, plasmids are subject to modification by the bacterial cell especially in recA strains, such as SL1344 and BJ66 used in this study. The recA locus encodes a recombinase that may delete regions of the plasmid containing the lux operon and the β-lactamase. Therefore, Salmonella recovered from cells in culture were plated both in the presence or absence of carbenicillin, and were imaged to determine the frequency at which bioluminescence was lost. All colonies recovered from gentamicin-treated, lysed HEp-2 cells and macrophages were ampicillin resistant (Amp^r) and bioluminescent. Therefore, lux genes appeared not to be lost during co-culture with mammalian cells.

Colonies were assayed for luminescence by visual inspection in a dark room. Five transformants were identified as having high levels of luminescence. Three of these, one each from the SL1344, BJ66 and LB5000 strains, were selected for subsequent experiments. They were termed SL1344lux, BJ66lux and LB5000lux, respectively.

C. Example 2

Invasive Potential Of Normal And Transformed Salmonella

The invasive potential of six strains of Salmonella (SL1344lux, LB5000lux, BJ66lux, SL1344, LB5000 and

BJ66) was determined using two types of bacterial adherence and entry assays. Colony-forming units (CFU) assays were performed essentially as previously described (Finlay and Falkow, 1989, *Mol. Microbiol.* 3:1833–1841) with modifications (Lee, et al., 1990, *PNAS* 87:4304–4308). Bioluminescence assays were performed essentially like the CFU assays, except that the number of cells was quantitated using bioluminescence, as opposed to CFUs.

Briefly, HEp-2 cells and primary murine peritoneal macrophages were seeded into 24-well tissue culture dishes at 1×10^5 cells per well in RPMI (Gibco/BRL, Grand Island, N.Y.) supplemented with 20 mM glutamine (Gibco/BRL) and 5 fetal calf serum (Hyclone, Logan, Utah). Twenty four hours (HEp-2) or seven days (macrophages) after cell seeding, bacteria from static cultures (see "Materials and Methods", above) were inoculated at 1×10^6 (multiplicity of infection (m.o.i.) of 10) or 1×10^7 (m.o.i. of 100, columns on right in FIGS. **2**B, **2**C, **2**D, and **2**E) organisms per well and centrifuged onto the cell monolayer for 5 minutes at 1000 rpm (185×g) in a Beckman clinical centrifuge (Beckman Instruments, Columbia, Md.). The medium was replaced with RPMI medium (Gibco/BRL) either with (entry assay) or without (adherence assay) gentamicin (100 mg/ml). The co-cultures were incubated for a total of 3.5 hours at 35° C. in 5% CO_2.

Gentamicin in the incubation medium kills bacteria that had not been internalized by the HEp-2 cells, including those adhering to the surfaces of the HEp-2 cells. Accordingly, the signal in adherence assays (without gentamicin) represent both adherent and internalized bacteria, whereas the signal in entry assays (with gentamicin) represent only internalized bacteria.

Adherence and entry were assayed by imaging luminescent bacterial cells at three timepoints—1.5, 3.0 and 3.5 hours post inoculation. Prior to imaging at the first timepoint, the cell monolayer was washed three times with phosphate-buffered saline (PBS) to remove unattached bacteria and a fresh aliquot of RPMI medium was added. Luminescence was recorded using a 30 second exposure. Images at the second and third timepoints were obtained using a similar exposure, but without first washing the cells.

Data recorded at the last timepoint, displayed as pseudocolor luminescence images superimposed over gray scale images of the culture dish wells, are shown in FIG. **2**A. The cell types, Salmonella strains, and usage of gentamicin are indicated in the FIGURE. The data are also summarized as relative intensity of photon counts in the graphs in FIGS. **2**B and **2**D.

Following imaging at the 3.5 hour timepoint, the tissue culture cells were washed three times with PBS and lysed with 0.20s "TRITON X-100" in PBS. Adherent and/or intracellular bacteria, released by lysis, were plated on LB- or LB-carbenicillin agar plates and incubated for 18 h at 35° C. The number of bacteria released from each well was determined by counting the number of colony forming units (CFU, Finlay and Falkow, 1989, *Mol. Microbiol.* 3:1833–1841., Lee, et al., 1990, *PNAS* 87:4304–4308). These data are represented as the total bacterial colonies per ml recovered from co-culture after incubation for 3.5 h with or without gentamicin, and are summarized in the graphs in FIGS. **2**C and **2**E.

Data from both the bioluminescence and CFU assays indicate that (i) Salmonella transformed with the lux genes have an infective potential similar to that of the parent lines, and (ii) luminescence detection and CFU determination yield comparable estimates for the invasive potential of the two Salmonella strains in HEp-2 cells and macrophages. The ratio of bioluminescence to CFU was lower in macrophage cultures, possibly due to the subcellular compartment in which the Salmonella enter macrophages.

D. Example 3

In vitro Luminescence of Transformed Salmonella

10 μl of four 10-fold serial dilutions (ranging from 10^6 cells to 10^3 cells per ml) of LB5000lux Salmonella were placed in four 100 μl glass capillary tubes (Clay-Adams div. of Becton Dickinson, Parsippany, N.J.). The bacterial suspensions formed columns of fluid in the tubes, with pockets of air at both ends. One end of each tube was sealed with critoseal (Clay-Adams). The medium in which dilutions were made was saturated with O_2 through exposure to air.

The tubes were wrapped with clear plastic wrap and luminescence was determined by imaging for 30 seconds as described above. An exemplary image is shown in FIG. **3**A. Four tubes are pictured. They contained (from top to bottom) 10^6, 10^5, 10^4 and 10^3 Salmonella cells/ml (10^4, 10^3, 10^2 and 10 cells/tube). Luminescence could be detected in suspensions containing as few as 10^4 cells/ml (100 cells). The luminescence is confined, however, to air/liquid interfaces, suggesting that the luminescence reaction requires relatively high levels of oxygen. Since many of the cells are presumably in the fluid column and not at the air/fluid interfaces, the data suggest that the luminescence in the capillary tubes shown in FIG. **3**A arises from considerably fewer than the total number of cells in each tube.

E. Example 4

In vitro Detection of Luminescence through Animal Tissue

Micro test-tubes, constructed from glass capillary tubing with an internal diameter of 3.5 mm, containing serial dilutions of LB5000lux Salmonella were prepared essentially as described in Example 3, above. In the present example, however, the bacterial suspensions contacted the sealed end of the tube and were exposed to air only at the upper end. The tubes were placed in a translucent plastic scintillation vial and surrounded by one of the following animal tissues: chicken breast muscle, chicken skin, lamb kidney or lamb renal medulla. All tissues were obtained from the meat department of a local supermarket (Safeway, Mountain View, Calif.).

A diagram of a vial containing a capillary tube surrounded by tissue is shown in FIG. **5**. The vial 1 is approximately 1.4 cm in diameter and includes a cap 2. The vial is coated with an opaque material (i.e., black tape) along its upper portion 3. Animal tissue 4 is placed in the vial such that it extends from the bottom of the vial to just above the bottom edge of the opaque coating 3. The micro test-tube 5 is sealed at the bottom by a plug 7 (i.e., a crytoseal plug), and is centered radially in the vial, with the plugged end of the tube touching or in close proximity to the bottom of the vial. The bacterial suspension 6 extends approximately 1 cm upward from the bottom of the tube.

Photons emitted from vials with and without tissue, and with and without bacteria, were counted using a liquid scintillation counter (model 1219 Rackbeta, LKB/Wallac, Gaithersburg, Md.) with the fast coincidence discriminator disabled.

Controls without tissue were assayed by placing the bacterial suspension directly in the scintillation vial. All experiments were performed in triplicate.

In each experiment, the vials were counted two to three times, rotating the vial 900 between each count, to control for effects of possible tissue thickness inconsistency. No significant differences were detected.

The results are summarized in TABLE I, below.

TABLE I

TRANSMISSION OF PHOTONS THROUGH TISSUE

Sample	Chicken skin	Chicken muscle	Lamb kidney	Lamb medulla
Vial alone	2.1×10^4	1.3×10^4	1.0×10^4	1.0×10^4
Tissue alone	N.D.	1.5×10^4	9.4×10^3	8.5×10^3
Tissue and LB5000lux*	2.7×10^5	2.3×10^5	1.6×10^4	1.5×10^5
LB5000lux* alone	2.0×10^6	1.7×10^6	4.8×10^6	4.8×10^6

Counts are averages of triplicate measurements, tissue path length was 1 cm. * – 1×10^7 cells.

The signal for 1×10^3 LB5000lux in kidney tissue was at or near background levels using the photomultiplier tubes (PMT) in the scintillation counter. The background in this type of detection is due to the dark current of the PMT and limits the studies to analysis of rather intense signals.

Bioluminescence from approximately 1×10^7 LB5000lux was detectable through 0.5 cm of avian muscle, skin ovine renal medulla and ovine kidney. These results indicate that bioluminescence from the labeled Salmonella was detectable through animal tissues of variable opacity. Since oxygen was likely limited in the capillary tubes (as demonstrated in FIG. 3A), it is likely that fewer numbers of bioluminescent Salmonella could be detected through tissue than are indicated in this assay.

F. Example 5

In Vivo Detection of Bioluminescent Salmonella

To assess the availability of oxygen to Salmonella during infection, wild-type SL1344lux was inoculated into the peritoneal cavity (i.p.) of BALB/c mice. Photons emitted from the bacteria internally, and transmitted through the abdominal wall were externally detected and localized in anaesthetized mice using an intensified CCD camera 24 h after inoculation (FIG. 3B). Systematic Salmonella infections are thought to involve colonization of the lymph nodes, spleen, liver,. Ventral images of the mice infected by i.p. inoculation of wild-type SL 1344lux demonstrated transmitted photons over much of the abdominal surface, with foci of various intensities (FIG. 3B). These results were consistent with widespread colonization of the viscera, possibly including the liver and mesenteric lymph nodes, and indicate that the level of available oxygen in some tissues can be adequate foe external detection of bioluminescence from the labelled pathogen.

G. Example 6

Effect Of Human Blood On The Light Emission From Bioluminescent Salmonella

As demonstrated in the following example, fewer than ten (10) bacterial cells can be detected with an intensified CCD detector.

Two fold serial dilutions of Salmonella, strain LB5000, that had been transformed with a plasmid that conferred constitutive expression of the luciferase operon were plated in duplicate into 96 well plates. Dilutions were made in 30 μl of growth medium alone (indicated as LB5000) and with 30 μl of blood to determine the effects of blood as a scattering and absorbing medium on the limits of detection. Each dilution and the numbers of colony forming units (CFU) implied from plating samples from concentrated wells are indicated in FIG. 4. The relative bioluminescence for each well as determined by analysis of the image generated by the CCD detector is shown (FIG. 4). The signal in the more concentrated wells was off scale and the numbers are therefore not linear at higher concentrations.

H. Example 7

Detection of Orally-Administered lux Salmonella in Balb/c Mice

Balb/c mice were infected by oral feeding (Stocker, et al.) with a 50 μl suspension of 1×10^7 virulent SL1344lux, non-invasive BJ66lux and low virulence LB5000lux Salmonella. The mice, 4–6 weeks of age at the time of infection, were imaged daily with 5 minute integration times (photon emission was measured for 5 minutes). Prior to imaging, the mice were anesthetized with 33 μg/kg body weight nembutal.

Representative images are shown in FIGS. 6A, 6B, and 6C. At 24 hours post inoculation (p.i.), the bioluminescent signal localized to a single focus in all infected animals (FIGS. 6A, 6B, and 6C). Bioluminescence disappeared in all animals infected with the low virulence LB5000lux by 7 days p.i. (FIG. 6A). In BALB/c mice infected with the wild-type SL1344lux, bioluminescence was detected throughout the study period, with multiple foci of transmitted photons at 8 d. In these animals, the infection frequently spread over much of the abdominal cavity (FIG. 6C). In one-third of these animals, transmitted photons were apparent over much of the abdominal area at 8 d, resembling the distribution of photons following an i.p. inoculation (see FIGS. 3B and 6F). The spread of infection by BJ66lux was more variable, but the infection typically persisted and remained localized at the initial site (FIG. 6B).

After infection of resistant BALB/cx129 mice with wild-type SL 1344lux, the bioluminescent signal remained localized and persistent in a group of 10 mice throughout the study period. This result was in contrast to the disseminated bioluminescence observed in SL1344lux-infected susceptible mice ($lty^{r/s}$) (see, Example 9 and FIGS. 8A and 8B), but resembled the persistent infection of susceptible BALB/c mice with the less invasive BJ66lux. As a control, Salmonella were cultured from persistently infected resistant BALB/cx129 mice, and 80–90% of the colonies recovered after 8 d were Amp^r. Of these, more than 90% were bioluminescent, suggesting that observed differences were not due to significant loss of lux plasmid, but rather were due to real differences in pathogenicity of the bacterial strains.

I. Example 8

Detection Of Infection Following I.P. Inoculation With A Virulent And A Low Virulence Strain Of Salmonella

Balb/c mice were infected with either virulent (SL1344lux) or low virulence (LB5000lux) Salmonella by intraperitoneal (i.p.) inoculations of 1×10^7 bacterial cells in a 100 μl suspension, without simultaneous injection of air.

At 32 hours post injection (p.i.), the mice were anesthetized and imaged as described above. The results are shown in FIG. 7. Widespread infection is evident in the two mice in the left part of FIG. 7, infected with the virulent SL1344lux strain. In contrast, little, if any, luminescence is

detected in the mice on the right, injected with the low virulence LB5000lux strain.

J. Example 9

Detection of Systemic Infection in Resistant Mice Following Oral Inoculation with Salmonella

Resistant 129×Balb/c ($Ity^{r/s}$) viable mice were infected by intragastric inoculation of 1×10^7 SL1344lux Salmonella. The bacteria were introduced through an intra-gastric feeding tube while under anesthesia. The animals were imaged daily for 8 days post injection (d.p.i.).

Results are shown in FIGS. **8**A and **8**B. Mice, in triplicate, were infected and imaged daily for 8 days. Exemplary images for day 1 (FIG. **8**A) and day 8 (FIG. **8**B) are shown. These data indicate that mice resistant to systemic Salmonella infection have a localized chronic infection in the cecum, but that the infection does not spread into the abdominal cavity.

K. Example 10

Post-Laparotomy Imaging following Oral Inoculation with Salmonella

Laparotomy was performed following oral inoculation of Salmonella to precisely localize the luminescent signal within the abdominal cavity, and to compare this localization with that obtained using non-invasive imaging. The animals were inoculated as described in Example 9. After a selected period of time, typically seven days, the mice were anesthetized and externally-imaged, as described above. An exemplary image is shown in FIG. **9**A. After external imaging, the peritoneal cavity was opened and the animals were imaged again, as illustrated in FIG. **9**B. In some instances the mice were imaged a third time, following injection of air into the lumen of the intestine both anterior and posterior to the cecum (C) (FIG. **9**C). The mice were euthanized immediately after the final imaging.

In each case where a focal pattern of bioluminescence was observed in susceptible mice, early in infection after oral inoculation, photons originated almost exclusively from the cecum, while variations in the precise localization and intensity of focal bioluminescence were due to variable positioning of the cecum. The focal pattern of bioluminescence observed in infection-resistant BALB/cx129 mice similarly localized to the cecum. In contrast, such localization was not observed in animals infected i.p. with SL1344lux (FIG. **3**B). At late stages in infection-susceptible mice inoculated orally with the wild-type SL1344lux, bioluminescence was multifocal, however, additional foci of luminescence did not become apparent after laparotomy. In mice infected with the less-virulent LB5000lux, bioluminescence was not detectable at 7 d in any tissue or organ, even focally, after removal of the skin and peritoneal wall.

Bioluminescence was not detected optically in the spleen or bloodstream of any infected animal; bioluminescence from the liver was seen only at later stages of disease; and bioluminescence from the G.I. tract was restricted to the cecum early in the disease course. This pattern could be due to differences in the Aumbers of Salmonella in the different tissues, or lack of available oxygen. The Amp^r cfu present in homogenized organs of orally infected mice were quantified to evaluate the distribution of labelled Salmonella SL1344lux. Greater than 90 k of the amp^r bacterial colonies obtained from all analyzed tissues of SL1344lux-infected BALB/c mice at 7 d indicated total cfu from the liver, spleen, and lungs were in the range of 1.9×10^3 to $>1.0\times10^5$ without detectable photon emission, in vivo (TABLE II). In contrast, bioluminescence was detectable from the cecum and this tissue contained $>1.0\times10^8$ total cfu. No cLu were detectable in any tissue of the LB5000lux infected mice. These results suggest that 1×10^8 organisms in tissue is near the limit of detection at this emission wavelength using the current experimental system.

Oxygen is an essential substrate for the luciferase reaction, thus only Salmonella present in oxygenated microenvironments should be bioluminescent. The absence of bioluminescence from Salmonella in the anaerobic environment of the lumen of the G.I. tract is therefore predictable, and exposure of the intestinal lumen to air should reveal the presence of bacteria previously not detectable due to a lack of oxygen. In support of this view, one animal with detectable bioluminescence in the cecum alone excreted a faecal pellet that rapidly became bioluminescent upon exposure to air. This indication of non-luminescent, luciferase-expressing bacteria in the lumen of the intestine and the clear delineation of the aerobic and anaerobic zones in this tissue, suggested that injection of air into the lumen of the intestine would reveal the presence of additional bacteria. Injection of air into the lumen of the ileum and colon of another animal, with a similar pattern of bioluminescence, resulted in detectable photons near the injection sites (FIG. **9**). Last, when a third mouse with cecal bioluminescence was killed, bioluminescence quickly ceased. Air was injected at other tissue sites because of the lock of clear zones of aerobic and anaerobic environments.

TABLE II

Colony-forming units in homogenized tissue from mice infected with bioluminescent Salmonella

Strain	Tissue	Animal Number	Tissue Weight (mg)	Total cfu
SL1344lux	Liver	1	441	1.9×10^3
		2	778	2.5×10^4
	Spleen	1	218	1.2×10^4
		2	248	4.9×10^5
	Mesenteric lymph node	1	76	$>1.0\times10^6$
		2	46	$>1.0\times10^6$
	Lung	1	17	1.5×10^3
		2	69	2.7×10^3
	Cecum	1	351	$>1.0\times10^{8*}$
		2	422	$>1.0\times10^{8*}$

*. Photons emitted from bacteria at these tissue sites were externally detected.

L. Example 11

Post-Laparotomy Imaging Following I.P. Inoculation with Salmonella

Balb/c mice were infected by intraperitoneal inoculation of 1×10^7 Salmonella (SL1344lux) as described in Example 8. Exemplary images of one such animal are shown in FIGS. **10**A, **10**B and **10**C.

At 24 hours post-injection (p.i.), the animal was anesthetized and image for five minutes (FIG. **10**A). The peritoneal vacity was opened and the mouse was imaged again for five minutes (FIG. **10**B). The cecum was pulled to the left side, and the animal was again imaged for five minutes (FIG. **10**A).

The results demonstrate that the localization of infection sites obtained with non-invasive imaging correlates well with the sites as revealed upon opening the peritoneal cavity.

M. Example 12

Effects of Ciprofloxacin Treatment on Bioluminescence from SL1344lux Salmonella

To demonstrate the utility of in vivo imaging, an infected animal was treated with the antibiotic ciprofloxacin, which known to be effective against systemic Salmonella infections. Magalianes, et al., 1993, *Antimicrobial Agents Chemo.* 37:2293.

Experimental and control groups of Balb/c mice were orally inoculated with SL1344lux. At 8 days p.i., mice in the experimental group were injected i.p. with 100 mg of ciprofloxacin hydrochloride (3mg/kg body weight; Sigma Chemical Co., St. Louis, Mo.). Following treatment of the experimental group, animals from both groups were imaged (as above) at several intervals over a period of 5.5 h post treatment.

Representative images are shown in FIGS. **11B**, **11C**, **11D**, and **11E**. FIGS. **11B** and **11D** show composite images of representative animals from the control and treated groups, respectively, immediately before initiation of treatment of the experimental group. FIGS. **11C** and **11E** show composite images of the same animals 5.5 hours after initiation of treatment. Bioluminescence over the abdomen of the ciprofloxacin-treated animal was reduced to undetectable levels during this period of time, while bioluminescence in the control typically increased 7.5-fold. The total number of photons detected over the abdominal area were determined, normalized to the value at t=0, and plotted in FIG. **11A** with respect to time post-treatment.

The data demonstrate that methods and compositions of the present invention can be used to evaluate the effects of drugs on the spread of infection in vivo.

N. Example 13

Bioluminescent Reporter For Promoter Activity In Cultured Cells

In order to demonstrate how the promoter from HIV (human immunodeficiency virus) responds to viral infection over time, jurket cells transfected with a plasmid containing the HIV LTR (long terminal repeat, promoter) upstream of the coding sequence of firefly luciferase were infected with a laboratory isolate of HIV-1 (strain A111) using standard laboratory conditions and followed for a period of 7 days (d) for emission of bioluminescent light. After 24 h, 60 h, 96 h, and 7 d, a gray scale image of the plate was generated in low room light followed by collection of photons emitted from the cultured cells in complete darkness for a period of 10 min. A color pseudoimage representing the intensity of bioluminescent light was superimposed over the gray scale image of the plate (FIG. **12**). At 7 d post infection a clear signal is present in the duplicate cultures indicating high levels of replication (FIG. **12**). The images at different time points represent the same two wells. Advantages of this assay for HIV replication are that: i) temporal studies can be done in a minimum number of wells since the same wells are followed over time, ii) kinetics of replication can therefore be studied as a phenotypic characteristic of viral isolates, iii) samples of cells or supernatant do not have to be collected, iv) the level of viral replication is almost immediately apparent, v) the detection could be set up remotely limiting human handling, vi) antiviral drugs could be evaluated in culture with the above listed advantages.

O. Example 14

Assessing Promoter Activity In Tissues Of Transgenic Mice

Transgenic mice containing a construct composed of the regulatory portion of the HIV LTR (U3 region) upstream of the coding sequence of the firefly luciferase gene were generated and evaluated for the emission of photons after transmission through tissues. A diagram of the construct is shown at the bottom of FIG. **13**. Numbers along the construct indicate nucleotide positions relative to the start of transcription. Sequences matching known motifs of cellular transcription factors are indicated with the names of the factors. Transcription from the HIV LTR was activated in the right ear of each two animals with a single topical application of dimethyl sulfoxide (DMSO). The animal on the left in FIG. **13** was given 150 μl of an aqueous solution of the substrate luciferin (50 mg/ml) via intraperitoneal (i.p.) injection. The animal on the right was not given substrate. 20 min. post treatment with subs:rate the animals were imaged as described for the plate in FIG. **12** with a 20 min. integration time. The color pseudoimage indicates light emission over the right ear (B) of the animal on the left, and not from the uninduced ear (A) or the animal that was not given substrate (C,D). This is the first demonstration of monitoring promoter activity in a living adult animal and demonstrates the relatively tight regulation of the LTR with DMSO induction. This technology allows for the temporal and spatial analyses of transcriptional activity in living animals.

P. Example 15

Topical Delivery Of Substrate To Dermal Cells In Transgenic Animals

In order to optimize delivery of substrate, the substrate was topically delivered to dermal cells. The HIV LTR was induced in the skin of mice with twice daily treatments of DMSO over the entire surface of the back and the right ear for two consecutive days. Substrate was applied to the skin in solutions prepared in DMSO. Concentrations included 200 mM, 100 mM, 50 nM, 25 mM, and 12.5 nm. 5 μl of each concentration were spotted, in quadruplicate, on the backs using a multichannel pipette with the highest concentrations near the head. 5 μl of the 50 mM solution was applied to each ear. 2 min. after application the animal was imaged as described in FIG. **12**. The bioluminescent response appeared increase linearly over the concentrations from 12.5 mM to 100 mM (FIG. **14**). Bioluminescence from spots containing 200 mM luciferin was roughly equivalent to that from the 100 mM spots, solutions of luciferin containing water, in contrast, resulted in no detectable bioluminescence (FIG. **14**). Solutions of 25% H_2O in DMSO to 100% H_20 were tested.

Q. Example 16

Induction Of Bioluminescence In Ears Of Transgenic Animals By Topical Luciferin Delivery

The experiment of induction of luciferase expression in ears and systemic luciferin delivery of Example 14 was repeated with topical administration of substrate in 100%

DMSO (FIG. 15). Signals from the ears were uniform and had greater intensity than with systemic luciferin delivery. See, FIGS. 13 and 15. Peak light emission was observed immediately after topical treatment compared to 20–30 min. after systemic administration of substrate.

R. Example 17

Unilateral Induction Of Luciferase Expression In Transgenic Mice

The left half of the shaved dorsal surface of the transgenic animals and the left ear were treated twice daily for two days with DMSO to activate expression of the HIV-1 LTR. Luciferin was applied topically over the entire surface of the back and both ears, and animals were imaged immediately after addition of substrate. Unilateral emission of bioluminescence corresponding to the induced region was observed (FIG. 16).

S. Example 18

Bioluminescence Detectable In Internal Tissues Of Transgenic Animals

Bioluminescence was detectable from the abdomens of animals treated with DMSO on one ear only. This signal is assumed to be due to ingestion of DMSO during grooming (FIG. 17).

T. Example 19

Localization Of Internal Bioluminescence In Transgenic Mice

Animals demonstrating signal from the abdomen were laprotomized and imaged. Bioluminescent signal localized to the colon in 4 of 4 animals studied and in the animals shown in FIG. 18 was tightly localized to a region of the colon about 1 cm in length. In the other animals the entire colon appeared to emit bioluminescent light.

U. Example 20

Expression Of The HIV-LTR In Neonatal Transgenic Mice

As the demonstrated in the following experiment, the HIV-LTR is differentially expressed through development.

4 d old transgenic mice were given intraperitoneal injections of luciferin in aqueous solution (15 μl at 50 mM), and imaged with integration times of 20 min. In the absence of any known inducing agent or treatment, bioluminescent signal indicative of expression of luciferase form the HIV LTR was apparent as a diffuse signal over much of the surface of the animal with more intense signal originating from the developing eye and extremities (FIG. 19). These data demonstrate that the LTR is inherently active in neonatal transgenic mice, and may be expressed to a greater level in the eyes and other locations.

All references are hereby incorporated in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 1

<210> SEQ ID NO 1
<211> LENGTH: 59
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Multiple Cloning Site of the lux pCGLS1 plasmid

<400> SEQUENCE: 1

ccaagcttgc atgcctgcag gtcgactcta gaggatcccc gggtaccgag ctcgaattc 59

What is claimed is:

1. A method for detecting a promoter-induction event in an animal, said method comprising the steps:

(a) triggering the event in a transgenic animal having a promoter responsive to such event and a heterologous gene encoding a light-generating protein under control thereof, and

(b) measuring with a photodetector device, photon emission through opaque tissue from expressed light-generating protein in the animal.

2. The method of claim 1, where the animal is a mammal.

3. The method of claim 1, where the mammal is a mouse.

4. The method of claim 1, where the animal is placed in the detection field of said photodetector device, and is maintained in an immobilized condition during the measuring of photon emission.

5. The method of claim 1, further comprising:

repeating step (b) at selected intervals,

wherein said repeating is effective to detect changes in the level of promoter induction in the animal over time.

6. The method of claim 1, where said detecting of a promoter induction event includes detecting the localization of the promoter induction event, said method further comprising,

(c) constructing an image of photon emission,

wherein said image shows the localization of the promoter induction event in said animal.

7. The method of claim 6, said method further comprising:

repeating steps (a) through (c) at selected intervals,

wherein said repeating is effective to track the localization of the promoter induction event in the animal over time.

8. The method of claim 6, further including the steps of

(d) acquiring a reflected light image of the subject; and

(e) superimposing said image of photon emission on said reflected light image to form a composite image,

where said acquiring may be performed either before or after said constructing.

9. The method of claim 1, wherein said measuring is carried out with an intensified charge-coupled photodetector device.

10. The method of claim 1, wherein said measuring is carried out with a reduced noise photodetector device.

11. The method of claim 1, wherein the light-generating protein is a bioluminescent protein.

12. The method of claim 11, wherein the luciferase is a prokaryotic luciferase.

13. The method of claim 11, wherein the luciferase is a eukaryotic luciferase.

14. The method of claim 13, further comprising, prior to said measuring, administering to the animal a substrate for said luciferase.

15. The method of claim 1, where said measuring is carried out using fiber optic cables.

16. The method of claim 11, where said fiber optic cables terminate in a tightly-packed array.

17. The method of claim 12, where said detection field is a surface defined by said tightly-packed array.

18. The method of claim 11, where said fiber optic cables detect light from a limited defined region of the subject.

19. The method of claim 1, where said measuring consists of measuring photon emission from within the subject with a photodetector device located outside of the subject.

20. The method of claim 1, where photons which make up said photon emission are visible light photons.

* * * * *

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Contag and Zhang

Serial No.: CPA of 09/464,795 Art Unit: 1632

Filing Date: 16 December 1999 Examiner: R. Shukla

Title: NON-INVASIVE EVALUATION OF PHYSIOLOGICAL RESPONSE IN A MAMMAL

DECLARATION OF DAVID B. WEST, PhD
PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, David B. West, hereby declare as follows:

1. I received my Bachelors of Science Degree in Biology from the University of Washington in 1977; and my Doctorate of Philosophy Degree in Physiology and Psychology in 1984 from the University of Washington.

2. I am currently the Senior Director of Preclinical Reserach at Xenogen Corporation and have held this position since April 9, 2001. Before joining Xenogen, I worked as Director of Mouse Genetics at Pfizer, Incorporated. Additional details regarding my background and qualifications can be found in the accompanying copy of my *Curriculum Vitae*.

3. I have reviewed pending Patent Application Serial No. 09/464,795 for "NON-INVASIVE EVALUATION OF PHYSIOLOGICAL RESPONSE IN A MAMMAL " by Contag and Zhang, (hereinafter "the specification") including pending claims 38, 40, 41, 43, 45, 46, 49 and 65-68. I have also reviewed (1) the Final Office Action dated September 13, 2001; (2) Cameron (1997) *Molecular Biotechnology* 7:253-

265; and (3) Cui et al. (1994) *Transgenic Research* 3:182-194. Therefore, I am familiar with the issues raised by the Examiner.

4. I understand that pending claims 38, 40, 41, 43, 45, 46, 49, and 65-68 are directed to transgenic mice and methods of using such mice. In particular, I understand that the transgenic mice comprise a panel of expression cassettes. Each expression cassette of this panel includes a control element from a stress-inducible gene operably linked to a sequence encoding a light-generating protein. Similarly, I understand that there are methods of using such mice to determine the effect of an analyte on gene expression.

5. In December of 1999, when the specification was filed, a typical scientist working the field of transgenic animals had a Ph.D. in the Biological or Chemical Sciences and two to five years of relevant experience. I will call such a person a "typical scientist."

6. When the specification was filed, it clearly conveyed to a typical scientist that the inventors had in their possession the invention of the claims (as set forth in paragraph 4, above). By "in their possession," I mean that the inventors contemplated transgenic mice comprising a panel of expression cassettes, wherein the panel comprises at least two different expression cassettes, each having a different stress-inducible control element operably linked to sequence encoding a light-generating polypeptide, and that they had, using the specification and information available to a typical scientist, a practical way of making and using such transgenic mice. Thus, I believe that a typical scientist would have understood the specification clearly described all of the various aspects of the claims and enabled a typical scientist to make and use the invention as set forth in the pending claims. I base this belief on the facts set forth below.

7. First, at the time the specification was filed, it was widely known how to construct expression cassettes generally. With regard to expression vectors comprising control elements from stress-inducible promoters operably linked to a sequence encoding a light-generating polypeptide, such methods are described in detail in the specification, for example, in Section 3.1.0 of the specification. Therefore, it is my opinion that construction of a panel of expression cassettes as set forth in the claims would have been routine to a typical scientist working in this area in view of the teachings of the specification.

8. Second, it would have been clear to a typical scientist that the inventors had in their possession the various polynucleotide components of the expression cassettes. Control elements derived from stress-inducible genes were known and clearly set forth in the specification at the time of filing. (See, Section 3.1.1 starting on page 35

of the specification). Similarly, the specification clearly describes sequences encoding light-generating proteins. (See, Section 3.2.0 starting on page 58 of the specification). Thus, it is my opinion that light-generating polypeptide-encoding sequences operably linked to control elements derived from stress-inducible genes of the expression cassettes of the claims are fully described in the specification.

9. Third, it would have been plain to a typical scientist from the specification that the inventors were in possession of an operative way of making the claimed transgenic mice. The specification describes methods of making transgenic animals on page 59, line 28 to page 60, line 8 and in the references cited therein. At the time the application was originally filed, such methods were routine to the typical scientist. Indeed, methods of introducing multiple expression constructs, each with their own separate promoter, to create transgenic founders are described in the art. (See, *e.g.*, Jankowsky et al. (2001) *Biomol Eng* 17(6):157-165, copy of the Abstract attached hereto). Also routine at the time of filing were methods of assaying if a sequence from an expression cassette had been integrated into a host mouse's genome and, if so, where such integration occurred. Such assay methods include, but are not limited to, PCR, Northern and/or Southern blotting (for example of particular tissues) as well as *in situ* hybridization and/or imaging techniques.

10. Fourth, a typical scientist would have known that the inventors were in possession of operative methods of using these transgenic mice, for example, to determine the effect of an analyte. The evaluation of whole transgenic animals having light-reporter systems is described on line 29, page 60 through line 6, page 61 of the specification. It is also my opinion that applying these methods of evaluation to the claimed transgenic mice and methods of using these mice would have been routine to one working in this area in view of Applicants teachings.

11. It is further my opinion that one skilled in the art would understand from the specification that the claimed transgenic mice could be made using techniques described in the specification or known at the time of filing. (See, *e.g.*, page 59, line 28 to page 60, line 8). Further, the specification discusses how to prepare transgenic animals and how to performing imaging experiments on these animals, etc. Thus, I believe that, based on the application and level of skill in the art, one working in this field would be able to make and use the claimed transgenic mice.

12. It is further my opinion that Cameron and Cui are not relevant to the subject matter claimed in the application. Cameron is directed primarily to transgenic livestock. (See, Cameron, Abstract). Further, the issues raised in Cameron regarding poor levels of expression are not relevant to the claimed invention for a variety of

reasons. First, transgenic mice containing the claimed expression cassettes can be readily assayed for expression levels and only those animals exhibiting the desired expression levels can be used. (See, also, paragraph 9 above). Second, leaky expression is not a major issue in the practice of the present invention -- where the expression cassettes integrate is irrelevant so long as expression of the light-generating protein is inducible via a stress-inducible control element. (See, also, paragraph 9 above). For its part, Cui is not relevant to the claimed invention because it is not directed to the use of light-generating proteins as *in situ* reporters. (See, Cui, page 183). Thus, I believe that one working in this field would have no reason to apply this information to the claimed invention. Accordingly, I do not believe that Cameron or Cui to be relevant to the claimed invention.

13. Therefore, taken as whole, the specification unambiguously conveyed to a typical scientist that the inventors contemplated including a panel of expression cassettes in a transgenic mouse comprising the stress-inducible control element operably linked to light-generating polypeptide-encoding sequence as disclosed in the specification. The inventors also had in their possession an operative way of using these transgenic animals to evaluate the effect of analyte in a whole animal. In sum, based on the disclosure of the specification and the level of knowledge of a typical scientist regarding expression cassettes, transgenic animals and assays for integration available at the time of filing, I believe that the specification as filed clearly conveys that the applicants had invented the expression cassettes and methods as set forth in the claims.

14. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

May 8, 2002
Date

David B. West, Ph.D.

CURRICULUM VITAE

David B. West, Ph.D.

PERSONAL

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Citizenship: United States
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Visiting Scientist
Lawrence Berkeley National Laboratory
Berkeley, CA

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Oakland, CA 94618
TEL: 510-339-2912

EDUCATION

1983 Ph.D. in Physiology/Psychology
University of Washington, Seattle, WA
Dissertation Title: Abnormal Perinatal and Infant Nutrition as a Cause of Obesity: Development of an Animal Model

1976 B.S. Cellular and Molecular Biology,
Cum Laude and Phi Beta Kappa
University of Washington, Seattle, WA

PROFESSIONAL EXPERIENCE

April 2001 – Present	Sr. Director of Preclinical Research Xenogen Corporation Alameda, CA
June 2000 – Jan. 2001	Director of Mouse Genetics Pfizer Global Research and Development Alameda, CA
May 1998 – June 2000	Director of Mouse Genetics Parke-Davis Laboratory for Molecular Genetics Alameda, CA
June 1999 – Present	Visiting Scientist Lawrence Berkeley National Laboratory Department of Molecular Medicine
May 1998 - Present	Adjunct Professor Pennington Biomedical Research Center
Mar. 1997 - Mar. 1998	On 75% time Academic Leave Participated in start-up of gene/Networks, Inc. A biotechnology company.
Sept. 1996 - 1998	Professor, Pennington Biomedical Research Center and Adjunct Professor in School of Veterinary Medicine and School of Medicine, Louisiana State University
Feb. 1991 - 1996	Associate Professor Pennington Biomedical Research Center Louisiana State University

Adjunct Associate Professor
Department of Physiology
School of Veterinary Medicine
Louisiana State University

Adjunct Associate Professor
Department of Physiology
Louisiana State University
School of Medicine
New Orleans, LA
(Effective 1993)

1987 - Feb. 1991	Assistant Professor of Physiology and Internal Medicine Eastern Virginia Medical School Norfolk, VA Staff Scientist Veterans Administration Medical Center Hampton, VA
1986 - 1987	Adjunct Assistant Professor of Biology Vassar College Poughkeepsie, NY
1984 - 1986	Individual NIH Postdoctoral Fellow Sponsor: Greenwood, M.R.C. Vassar College Poughkeepsie, NY
1977 - 1983	NIH Predoctoral Fellow in the Departments of Physiology and Psychology University of Washington Seattle, WA

RELATED PROFESSIONAL EXPERIENCE

1984 - 1987	Writer, Medical Literature Review Corporation St. James, NY

RESEARCH FUNDING

March, 1998- February, 2002	National Institutes of Health RO1 #DK53393 An obesity locus on mouse chromosome 7 Estimated total direct costs for 4 years: $1,002,628 Approximately 40% of project subcontracted to Dr. Dabney Johnson, Oakridge National Laboratory
1994 - Sept, 2001	National Institutes of Health RO1 #DK45895 Metabolic and genetic markers for dietary obesity Current funding period September 1, 1997 - August 31, 2001 Estimated total direct costs for 4 years: $1,160,521
Jan, 1998 - Dec, 1999	National Cattleman's Beef Association Co-PI with James P. DeLany Effects of conjugated linoleic acid on metabolism in the mouse.

Estimated total direct costs for 1 year: $75,000

1991 - 1996
National Institutes of Health R29 #DK44446
Nutrition and hypertension: Role of hyperinsulinemia

1991 - 1992
Pew National Nutrition Program
Fellowship for Faculty Scholars in Nutrition

1989 - 1990
Diabetes Center of Eastern Virginia
Grant in Aid.
Insulin resistance and dietary obesity: Role of altered renal function in the development of obesity-related hypertension

1987 - 1988
Eastern Virginia Medical School
Institutional Research Grant
Obesity and Hypertension

1985 - 1988
National Institutes of Health ROl #HD12637
Co-Principal Investigator with Greenwood, M.R.C.,
Nutrition and adipocyte growth and development

1985 - 1987
Hoffmann-La Roche Inc.
Research Contract
Peptides and feeding behavior

1985 - 1987
New York Heart Association
Grant-in-Aid
Co-Principal Investigator with Greenwood, M.R.C.,
Altered blood flow and metabolism in obese fa/fa rats

1984 - 1986
National Institutes of Health
Individual Postdoctoral Fellowship #AMO7332
Early nutritional factors in the etiology of obesity

RESEARCH INTERESTS

Molecular genetics of obesity
Molecular genetics of complex disorders
Obesity and hypertension
Early nutrition in the etiology of obesity
Adipose tissue physiology and metabolism

ACADEMIC RESEARCH IN PROGRESS

NIH-funded: Genetic and metabolic basis of dietary obesity
Cloning an obesity gene on mouse chromosome 7

Other: Metabolic effects of conjugated linoleic acid

TEACHING EXPERIENCE

1987 - 1991 Eastern Virginia Medical School:
Physiology: Year 1 Medical Students

1984 - 1987 Vassar College:
Guest Lecturer, Endocrinology

Laboratory Instructor in Nutrition (Supervisor of a minimum of 3 independent students each year)
Guest Lecturer, Principles of Nutrition

University of Washington:
1982 Instructor, Surgical and Histological Techniques

1981 Teaching Assistant, Animal Learning Laboratory

1978 - 1980 Teaching Assistant, Surgical and Histological Techniques

TEACHING INTERESTS

Molecular Genetics
Nutrition
Physiology
Metabolism and Endocrinology

GRADUATE STUDENTS SUPERVISED

Gerald Thompson, Department of Kinesiology, Louisiana State University; Ph.D. awarded in July, 1996.
Dissertation: Post-exercise Hypotension in the Dog

Agatha Borne, DVM, Department of Physiology, School of Veterinary Medicine, Louisiana State University; Ph.D. awarded in May, 1998.
Dissertation: Interaction of Nitric Oxide and the Sympathetic Nervous System in the Control of Regional Vascular Resistance

MEMBERSHIPS IN SCIENTIFIC AND ACADEMIC SOCIETIES

American Association for the Advancement of Science
American Institute of Nutrition
American Physiological Society
North American Association for the Study of Obesity
Society for the Study of Ingestive Behavior

ACADEMIC HONORS AND AWARDS

1984 - 1986	NIH Individual Postdoctoral Fellowship Vassar College, Poughkeepsie, NY
1981	NSF Undergraduate Research Program Grant Co-sponsor with Stephen C. Woods, Ph.D. University of Washington, Seattle, WA
1980 - 1981	Graduate School Research Fund Grant Recipient (co-authored with Herman Samson, Ph.D.) Mechanisms of Satiety Hormones University of Washington, Seattle, WA
1977 - 1982	NIH Predoctoral Fellow University of Washington, Seattle, WA
1976	Bachelor of Science University of Washington, Seattle, WA Cum Laude and Phi Beta Kappa

NATIONAL PROFESSIONAL SERVICE

1997	Ad hoc member, NIH Special Study Section
1996	Ad hoc member, NIH Special Study Sections

Organizing Committee, 1998 Summer Faseb Conference on
Behavioral and Metabolic Subphenotypes in Obesity

1995 Organizing Committee, Annual Meeting for the North American Association for the Study of Obesity

Ad hoc member, NIH Special Study Section

1994 - 1996 Education Committee, North American Association for the Study of Obesity

1993: Ad hoc member Nutrition Study Section
CNRU Site Visit Team, National Cancer Institute

INSTITUTIONAL SERVICE

1993 - 1997 Institutional Animal Care and Use Committee
Pennington Biomedical Research Center

1990 - 1991 Chairman, Curriculum Committee
Eastern Virginia Medical School

1989 - 1990 Member, Curriculum Committee
Eastern Virginia Medical School

1988 - 1991 Institutional Animal Care and Use Committee
Veterans Affairs Medical Center, Hampton, VA

AD HOC REVIEWER

American Journal of Physiology
Appetite
Genomics
International Journal of Obesity
Journal of Nutrition
Journal of Clinical Nutrition
Journal of Clinical Investigation
Mammalian Genome
Metabolism
Obesity Research
Peptides
Physiology & Behavior

Proceedings of the National Academy of Science

EDITORIAL BOARDS

American Journal of Physiology (1993)

INVITED PRESENTATIONS

2000: "Mouse Models and Functional Genomics". Invited speaker at the Jackson Laboratory/Roche Laboratory symposium on Functional Genomics of Diabetes And Obesity. Palo Alto, CA.

1999: "Complex Genetics of Obesity". Invited speaker at 1999 Neuroscience Festival at the University of Cincinnati, Cincinnati, OH

Panelist: "Dietary fat and obesity" 1999 meeting of the Society for Experimental Biology, Washington D.C.

"Congenic Lines and the Deconvolution of Complex Genetics". Invited seminar speaker at the University of Oregon Health Sciences Center, Department of Neurosciences, Portland, OR

"Mouse Genetics/Genomics for Target Identification and Validation". Invited Speaker at the annual winter conference on Medicinal and Bio-organic Chemistry, Park City, UT

1998: "Genetic Basis of High-Fat Induced Obesity". Presentation at the 8th International Congress on Obesity, Paris, France

"Obesity QTLs in Rodents". American College of Sports Medicine. Symposium on Obesity, Orlando, FL

"Man-Mouse Synteny: A Paradigm for Unraveling the Complexity of Human Genetic Disorders". March of Dimes Symposium on Complex Human Genetic Disorders. Los Angeles, CA

"Dietary Fat and Gene Interactions". Pennington Biomedical Research Center Symposium on "Nutrition, Genetics and Obesity", Baton Rouge, LA

1997: "Genetics of Obesity in Animal Models: Relevance to Obesity Associated Hypertension. Presented at 1997 Experimental Biology

Symposium on Obesity and Hypertension. New Orleans, LA

1996: "Genetics and Physiology of Dietary Obesity in the Mouse" presented to: Department of Clinical Nutrition, University of Texas Southwestern Medical School, Dallas TX

Smith Kline Beecham, Welwyn Garden City, England

School of Agriculture, University of Nebraska, Lincoln NB

Symposium on Genetics of Obesity in Animal Models, Experimental Biology 1996 Annual Meeting, Washington, D.C.

2nd International Conference on Oils and Disease, University of Texas Southwestern Medical School, Dallas TX

Sixth Benjamin Franklin Lafayette Seminar on Mechanisms of Food Intake and Specific Appetites, Sponsored by Cornell University, Pennsylvania State University, and College de France, La Napoule, France

Third International Symposium on Obesity and NIDDM, Sponsored by The Clore Laboratory at the University of Buckingham, Buckingham England

Department of Nutritional Sciences, University of Illinois, Champagne-Urbana, IL

Biology Section, Oakridge National Laboratory, Oakridge, TN

Psychology Department, Florida State University, Tallahassee FL

1995: FASEB Summer Research Conference on Genetic and Behavioral Influences on Nutrient Metabolism and Obesity, Copper Mountain, CO. "Molecular Genetics of Dietary Obesity in the Mouse"

NIH, NIDDK Conference on Prevention and Treatment of Childhood Obesity, Bethesda, MD. "Molecular Genetics: Implications for Pediatric Obesity Research"

Annual Meeting of the North American Association
for the Study of Obesity, Baton Rouge, LA. "Dietary
Fat and Obesity: Genetic Models of Obesity in Animals"

CME Course on the Prevention and Treatment of
Obesity in Special Populations, New Orleans, LA.
"Genetics/Environment is the Primary Determinant
of Most Cases of Obesity"

1994: Symposium on the Molecular and Genetic Aspects of
Obesity, Pennington Biomedical Research Center
Baton Rouge, Louisiana "Genetics of Dietary Obesity"

Seventh International Congress on Obesity
Toronto, Canada
Round Table Discussant; Prevention of Obesity

Obesity, Diabetes, and Insulin Resistance:
Implications from Molecular Biology, Epidemiology,
and Experimental Studies in Humans and Animals
American Diabetes Association
Boston, Massachusetts
"Dietary Obesity, Insulin Resistance, and Hypertension, A Canine Model"

Visiting Scientist, Jackson Laboratory,
Bar Harbor, Maine "Molecular Genetics of Dietary
Obesity in the Mouse"

1993: Division of Cardiology
Obesity Training Grant Speakers Program
University of California
Los Angeles, California
"Genetics of Dietary Obesity in the Mouse"

Department of Nutrition
University of California
Davis, California
"Genetics of Dietary Obesity in the Mouse"

First Department of Internal Medicine
Gunma University School of Medicine
Gunma, Japan

"Genetics and Physiology of Dietary Obesity in the Mouse"

1992: Continuing Medical Education
Emory University
Obesity Update: Pathophysiology, Clinical Consequences, and Therapeutic Options
"Hypertension and Obesity"

1991: Fifth Benjamin Franklin/Lafayette Symposium on the Physiology of Appetitive Behavior
La NaPoule, France
"Dietary Obesity in Mice"

1989: FASEB Summer Research Conference
on Energy Metabolism.
Saxtons River, VT
"Adipose Tissue Blood Flow and Metabolism"

Annual meeting of the North American Association for the Study of Obesity
Washington, DC
"Animal Models of Obesity Associated Hypertension"

1988: Benjamin Franklin/Lafayette Symposium on the Physiology of Appetitive Behavior
La NaPoule, France
"Peptide Hormones and the Control of Food Intake"

Buckingham University
Symposium on Insulin and Obesity
Buckingham, England
"Regulation of Adipose Tissue Blood Flow by Insulin"

1987: Appetitive Seminar
Columbia University, New York, NY
"The Use of Short-acting Anorectic Agents for the Long-term Reduction of Food Intake"

Fifth Annual Virginia Nutrition Conference
"Role of Genetics and Adipocyte Development"

Harvard Medical School Continuing Education
Program on Treatment of Obesity: Diet,
Pharmacology, and Surgical Approaches
Cambridge, MA
"Cholecystokinin and Other Peptide Hormones"

1986: Department of Pharmacology
Hoffman La-Roche, Nutley, NJ
"Peptides and the Chronic Suppression of Food Intake"

Department of Psychology
State University of New York, Albany, NY
"Early Nutrition and the Development of Obesity"

Department of Nutrition
University of Georgia, Athens, GA
"Experimental Approaches in Animals to Study the Causes and Consequences of Obesity"

REFERENCES

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PUBLICATIONS

Iakoubova, O.A., Olsson, C.L., Dains, K.M., Choi, J., Kalcheva, I., Bentley, L.G., Cunanan, M., Hillman, D., Louie, J., Machrus, M. and West, D.B. Microsattellite marker panels for use in high-throughput genotyping of mouse crosses. In Press: *Physiological Genomics*.

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Co-expression of multiple transgenes in mouse CNS: a comparison of strategies.

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The introduction of two transgenes into one animal is increasingly common as transgenic experiments become more sophisticated. In this study we examine two strategies for creating double transgenic founders from a single microinjection. In the first approach, two constructs, each with its own promoter element, were coinjected into the pronucleus. In the second approach, both transgenes were cloned into one vector, separated by an internal ribosomal entry site (IRES), and placed under control of a single promoter. Both strategies save time and increase the percentage of double transgenic offspring over the standard method of mating single transgenic lines. However, despite high transgene copy numbers, the bicistronic lines did not show robust expression of either protein. Copy number and protein expression correlated much better in the coinjected lines, with expression levels in one line approaching that observed in some of our best single transgenic controls. Thus we recommend coinjection of individual plasmids for the generation of multiply transgenic founders.

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RELATED PROCEEDINGS APPENDIX

Appellants are not aware of any related proceedings. In as much as no decisions have been rendered by a court or the Board in this related case, no documents are submitted with this Appendix.